
**Development of a high pressure processing
inactivation model for hepatitis A virus and
its application in shellfish processing**

by

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Declaration of originality

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List of abbreviations

A_f	Accuracy factor
B_f	Bias factor
bp	Base pair
BSA	Bovine serum albumin
Ct	Cycle threshold
FCV	Feline calicivirus
HAV	Hepatitis A virus
HPP	High pressure processing
IMS	Immuno-magnetic separation
LogS	Survival ratio (logarithm of the remaining infectious fraction of HAV at a particular treatment time)
MNV-1	Murine norovirus
MPa	Megapascal
NBS	Newborn bovine serum
PBS	Phosphate-buffered saline
PFU	Plaque-forming units
ppt	Parts per thousand
PV	Poliovirus
qRT-PCR	Quantitative reverse transcription-polymerase chain reaction
R^2	Coefficient of determination
RFU	Relative fluorescence units
SS	Sum of squared residuals
TCID ₅₀	50% tissue culture infective dose
T_M	Melting temperature
×g	Relative centrifugal force

Abstract

Hepatitis A virus (HAV) has been responsible for many large outbreaks of illness throughout the world, often resulting from consuming raw or minimally cooked filter-feeding shellfish contaminated with human faecal effluent.

High pressure processing (HPP) is an alternative food preservation technique to heat, preserving the flavour, appearance and nutritional value of high quality foods, including oysters, often with extended shelf life. In this study, the effectiveness of HPP in inactivating HAV was assessed.

HAV, suspended in buffered tissue culture media containing either 15 parts per thousand (ppt) or 30 ppt salt (NaCl), was treated with 300, 400 and 500 MPa for between 60 and 600 s. A log-linear function was developed in Microsoft® Excel to model the kinetic inactivation data with the effects of NaCl, pressure and treatment time. The model can be used to predict HAV inactivation by interpolation at processing parameters not actually tested for in the laboratory.

For the model to be validated in oysters contaminated with HAV, methods for HAV extraction and purification from spiked oyster homogenate were first evaluated. Methods evaluated included the crude extraction method, modified from Kingsley and Richards (2003), and the PEG precipitation method, modified from the glycine-polyethylene glycol (PEG)-Tri reagent-poly(dT) extraction (GPTT) method described by Kingsley and Richards (2001). The PEG precipitation method achieved a mean recovery of 12.6%. With modification,

including the use of antibiotic/antimycotic treatment prior to assay, the recovery was improved by up to 27.3%. In comparison, the crude extraction technique, which did not include a virion concentration step, recovered on average more than 40% of the initial spiked titre, and was chosen as a reliable method to extract HAV from oysters for cell culture quantitation.

Commercially grown and harvested Pacific oysters (*Crassostrea gigas*) were contaminated with HAV by natural accumulation, when immersed for up to 24 h in seawater contaminated with 1.1×10^7 TCID₅₀/ml HAV. Infectious HAV was detected in only two of the six oysters tested, and less than 1% of the initial contaminating HAV titre was recovered in positive oysters, possibly due to the association of viruses and microalgae with oyster shells and aquarium surfaces throughout the trials.

A quantitative real-time reverse transcription PCR (qRT-PCR) method was developed as an alternative method for HAV detection in contaminated oysters to the infectivity assay. HAV was detected by qRT-PCR in all contaminated oysters, including those negative by infectivity assay. An immuno-magnetic bead separation technique was also developed, which additionally purified and concentrated virions, improving the sensitivity of detection by qRT-PCR.

The log-linear inactivation model was validated in homogenised oyster meat artificially inoculated with known titres of HAV. Salinity and temperature of samples were adjusted to that of buffered samples, while intermediate times and pressures were chosen for processing. The model tended to underpredict

inactivation in homogenised oyster samples; i.e. it was fail-safe. Inactivation tended to be greater in spiked oyster homogenate compared to pure culture in treatments at higher pressures (400 – 500 MPa). The validated model may be a useful reference for Australian oyster processors wishing to implement HPP into their post-harvest processing regime.

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Chapter 1: Literature review.

1.1 INTRODUCTION

All foodborne viruses that are detrimental to human health emanate from the human intestinal tract (Blackwell *et al.*, 1985). Faecal-oral transmission can occur by indirect routes or direct personal contact. Those foods and beverages susceptible to faecal contamination and lacking an intervention step such as cooking prior to consumption usually carry a greater risk of causing viral illness.

Foodborne viruses are considered to be responsible for the majority of foodborne illness in the United States of America (U.S.A.) by a wide margin (Mead *et al.*, 1999), but viruses are often the least or last studied in process development, and are not routinely tested in food and environmental samples due to technical and cost issues associated with their extraction, observation and culture.

Viruses differ greatly from the bacterial agents of foodborne disease. They have been described as extracellular organelles evolved to transfer nucleic acid from one cell to another (Harrison *et al.*, 1996). Viruses have no cellular structure and contain either RNA or DNA enclosed in a protein coat or capsid (Madigan *et al.*, 2000). The capsid functions as the primary protective barrier for the viral particle or virion. The capsid of some viruses is additionally enveloped in an outer lipid membrane. All human enteric viruses are non-enveloped, as enveloped viruses tend to be susceptible to adverse environmental conditions and are generally destroyed by the low pH and bile found in the gastrointestinal tract of humans (Adams and Moss, 2000).

The diameters of virus particles range between 25 to 300 nm, so most cannot be visualized with a light microscope. Furthermore, viruses are obligate intracellular parasites, meaning they can only replicate in a suitable living host cell, and not in the environment (Cliver and Matsui, 2002). However, the usually low infectious dose of enteric viruses (believed to be far less than 100 virions), means that even a small amount of contamination in food may result in a significant threat to public health (Jaykus, 2000; Cliver and Matsui, 2002). This is coupled with a high level of persistence in foods and the environment, with select enteric viruses being able to withstand conditions such as high acidity (Scholz *et al.*, 1989), freezing temperatures and reduced water activity without loss of infectivity for extended periods of time, as reviewed by Koopmans and Duizer (2004).

1.2 VIRAL ENTERITIS

Over the last several years there has been a growing awareness of the significant role viruses play in foodborne disease. While it is uncertain how many different viral diseases have been or can be spread by contaminated foods and beverages, the number of different viruses acknowledged as primary agents of foodborne illness is actually quite low. Noroviruses are now recognized as the most common cause of all foodborne disease in the U.S.A., estimated to be responsible for 23 million cases annually (Mead *et al.*, 1999). Approximately 40% or 9.2 million cases per year are estimated to be foodborne, and this number corresponds to 67% of all cases, 33% of hospitalizations and 7% of deaths due to foodborne illness annually (Mead *et al.*, 1999). Furthermore, noroviruses appear to be becoming increasingly virulent (Food Safety Network, 2005).

The incidence of hepatitis A in Australia and the U.S. is declining, but outbreaks continue to occur despite an increase in sanitation and hygiene standards (Grohmann and Lee, 2003). Due to the serious nature of the disease it can cause, hepatitis A virus (HAV) is usually ranked second on the list of important foodborne viruses, and is described as the only common vaccine-preventable foodborne disease in the U.S. (Fiore, 2004).

Human enteric viruses are transmitted by the faecal-oral route via contamination with human faecal matter. Foodborne transmission of human enteric viruses most frequently occurs in those foods requiring little or no intervention (*e.g.* heat processing) prior to consumption, and/or ready-to-eat foods that are prepared by a food handler immediately before consumption. As their name suggests, 'filter-feeding' shellfish, such as oysters feed by filtering small particles such as algae from the surrounding water. Oysters in particular can accumulate microorganisms to concentrations higher than that in the surrounding water. Fresh produce may also transmit enteric viruses following irrigation with contaminated water, or food may be contaminated by infected food handlers with poor personal hygiene. These foods may benefit from more stringent farming practices and improved education for food handlers, as well as an intervention strategy that would ensure the 'fresh' qualities of the food.

1.3 HEPATITIS A VIRUS

Hepatitis A virus (HAV) belongs in the genus *Hepatovirus*, within the family *Picornaviridae*. Seven distinct genotypes of HAV have been described (I to VII), with viruses from four genotypes (I, II, III and VII) isolated from humans, and viruses from the remaining three genotypes (IV, V and VI) classified as simian strains (Lemon *et al.*, 1992; Robertson *et al.*, 1992).

The HAV genome, of single-stranded, positive-sense (*i.e.*, translatable) RNA, is approximately 7.5 kb in length, and like all picornaviral genomes, consists of three parts: Two noncoding regions (NCR) and an open reading frame (ORF), which contains the regions P1 (for the capsid proteins VP1, VP2 and VP3), and P2 and P3 (for nonstructural proteins) (Cuthbert, 2001; Hollinger and Ticehurst, 1996; Rueckert and Wimmer, 1984). The nucleic acid is packaged within a protein capsid of icosahedral symmetry (faces of the capsid are equilateral triangles). The intact virion is approximately 27-30 nm in diameter without distinctive surface features (Cliver and Matsui, 2002; Büchen-Osmond, 2003) (Figure 1.1). As reviewed previously (Cliver and Matsui, 2002; Grohmann and Lee, 2003; Hollinger and Ticehurst, 1996), HAV possesses notable stability to environmental conditions, particularly to heat and drying, and is more resistant to low pH (pH 1.0), gamma rays, UV light and low levels of chlorine and ozone than other picornaviruses.

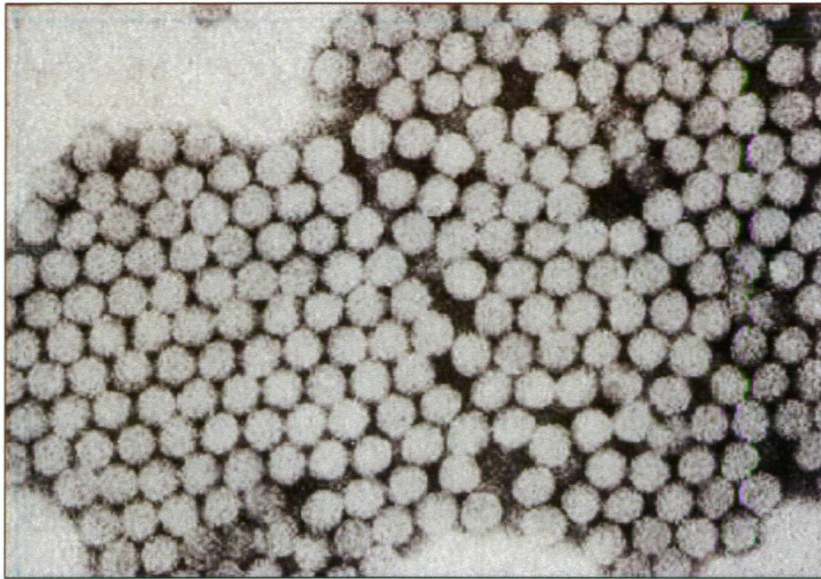


Figure 1.1. Electron micrograph of hepatitis A virus (HAV) virions.

Individual virions are 27-30 nm diameter.

(Source: Centers for Disease Control and Prevention (CDC),

http://www.cdc.gov/ncidod/diseases/hepatitis/slideset/hep_a/slide_3.htm)

1.3.1 Difficulties of laboratory study

Tissue culture is an effective method for the propagation and quantification of a variety of viruses, including those pathogenic to humans, but propagation of HAV *in vitro* using mammalian tissue culture cells can best be described as only 'moderately successful' (Koopmans *et al.*, 2002), especially for environmental isolates or wild-type strains which can prove exceptionally difficult to propagate.

Enumeration of viable viruses traditionally requires the development of plaques and cytopathic effects in infected tissue culture cells. Although virions are released from infected cells into the surrounding liquid medium where they may be recovered for further analysis, only a relatively small proportion of HAV is released from infected tissue culture cells (Hollinger and Ticehurst, 1996). Instead, most of the infectious virions remain in the cell cytoplasm (Bishop *et al.*, 1994). Tissue culture methods for analysing and enumerating HAV remain lengthy and labour-intensive, thereby encouraging the use of rapid detection methods including PCR techniques. However, PCR-based techniques, whilst considerably faster to perform, are compromised by insufficient sample purification prior to assay, the presence of food-related inhibitory compounds, and the detection of the sequence of interest in genetic material from any intact virion, whether it is infectious or not (Richards, 1999; Jaykus, 2000).

1.3.2 Illness and transmission of infection

Human HAV is shed exclusively in human faeces; therefore infection is initiated when the virus is ingested, commonly via contaminated water or food (Cliver and Matsui, 2002). Transmission may also occur by exposure to HAV-contaminated blood or blood products (Fiore, 2004). After penetrating and replicating in the intestinal epithelial cells, the virus infects the liver where replication also takes place inside hepatocytes (Cuthbert, 2001; Hollinger and Ticehurst, 1996). When an immune response is evoked, cytotoxic T cells destroy infected liver cells, severely disrupting regular body functions controlled by the liver (Cliver and Matsui, 2002). Following secretion of the virus from the liver in bile, hepatitis A virions are excreted from the body in faeces. Consequently, if treatment processes for human faecal effluent are inadequate to eliminate HAV prior to release into the environment, a significant risk may be posed to drinking and irrigation water, waters from which shellfish are harvested, and waters for recreational use (Cliver and Matsui, 2002).

The incubation period of hepatitis A averages 28-30 days, and during this time the virus is shed from the body. Virions continue to be shed in low numbers for up to 2 weeks following the onset of symptoms which include fever, loss of appetite, nausea and abdominal discomfort, often followed by jaundice lasting several days (Cliver and Matsui, 2002). In infants and children under five years of age, infection is often mild or asymptomatic. Immunity to hepatitis A is usually life-long and death is rare (Grohmann and Lee, 2003).

From 1987 to 1997 an average of 120,000 acute hepatitis A cases were estimated to occur annually in the U.S.A., but following the introduction of an effective vaccine in the mid-1990's, cases of hepatitis A have been decreasing since 1997, with almost 6,000 reported and 24,000 estimated cases of acute hepatitis A in 2004 (Centers for Disease Control and Prevention (CDC), 2006; Food Safety Network, 2005). The occurrence of hepatitis A in Australia has also declined over the last 30 years; however, the occurrence in some indigenous human communities remains high (Lin *et al.*, 2002). Between 1991 and 1999, the annual number of hepatitis A infections in Australia averaged 2,115, but in 2000, the number of hepatitis A cases declined by 48% from the previous year. This reduction was thought to be due to effective control measures such as vaccinations of susceptible populations and improvements in hygiene (Lin *et al.*, 2002).

Hepatitis A can cause particular public health problems in areas lacking proper sewage treatment facilities, as well as in locations where an adequate level of hygiene may be difficult to maintain, such as child care centres, prisons, and camp grounds (Grohmann and Lee, 2003). The disease is endemic in developing regions such as Southeast Asia, the Indian subcontinent and Africa (Koopmans *et al.*, 2002). In such areas, many children under the age of six acquire a mild or subclinical disease, and thus immunity to re-infection. Consequently, the occurrence of hepatitis A outbreaks in these areas is rare (Koopmans *et al.*, 2002). In developed countries, however, improved sanitary conditions have resulted in fewer cases of hepatitis A, but left large populations susceptible to a greater risk of large-scale epidemics (Conaty *et al.*, 2000, Lees, 2000).

1.3.3 Outbreaks of interest

In 1997, a highly publicised hepatitis A outbreak in Australia resulted from the consumption of contaminated shellfish (Conaty *et al.*, 2000). More than 400 hepatitis A cases, including one death, were reported following the consumption of infected oysters harvested from the Wallis Lakes area in New South Wales (N.S.W.), Australia (Conaty *et al.*, 2000). Whilst the precise cause of contamination in this outbreak is unknown, it was suggested that unusually high rainfalls resulted in a turbid flow of water to the oyster harvesting area. Heavy rain can widely distribute a source of contamination in a lake or estuarine area, and probably ensured the pollution was well dispersed almost lake-wide (Conaty *et al.*, 2000). This outbreak occurred despite no apparent breach of microbiological safety regulations, including a mandatory period of shellfish purification following harvest, industry safeguards of ceasing shellfish harvests following rainfall, and the regular testing of shellfish meat for indicator microorganisms (Conaty *et al.*, 2000).

In 1988, an outbreak of hepatitis A linked to contaminated raw clams in China killed 32 people and infected nearly 300,000 (Halliday *et al.*, 1991). This outbreak may well be the largest recorded viral food poisoning outbreak in the world to date. Clams were contaminated by the release of untreated human faecal effluent from a nearby residential area which had reported an epidemic of hepatitis A in the preceding months, and from boats dumping human waste overboard in the vicinity of harvesting areas.

In October and November 2003, a large hepatitis A outbreak originated in a restaurant in Pennsylvania, U.S.A. and received nationwide media coverage, as more than 600 people demonstrated symptoms consistent with hepatitis A infection, and three fatalities were recorded (Wheeler *et al.*, 2005). Green onions imported from Mexico and exposed to human faeces were confirmed as the vector, and were an ingredient in a mild salsa that was served to all patrons in the restaurant during the outbreak.

1.4 BIVALVE MOLLUSCAN SHELLFISH

Bivalve molluscan shellfish such as oysters, clams, mussels and scallops are soft-bodied animals protected by a shell that is divided in two halves (valves). Powerful adductor muscles near the hinge of each valve close the shell for protection, and open to allow feeding and respiration via the gills (Campbell, 1996). Bivalve molluscs are filter feeders, inducing a current of water (up to 20 L water per hour under ideal conditions) to pass over gills (Grohmann and Lee, 2003). As shellfish pump water, strands of mucous are continuously secreted, trapping food particles such as algae and microorganisms, which are then carried by cilia to the mouth area to be either ingested or eliminated as pseudofaeces (DiGirolamo *et al.*, 1970).

Filter-feeding shellfish are at particular risk of human enteric virus transmission. They are usually cultivated in estuaries where the sheltered waters contain high nutrient levels; however, these shallow inlet waters may also be contaminated with human sewage and other forms of pollution (Lees, 2000).

The microbiological profile of oysters is directly related to that of the surrounding water (Son and Fleet, 1980). Pathogenic microorganisms bioaccumulated by oysters are often detected in oyster flesh at concentrations greater than that in the surrounding water. Because oysters and other bivalve molluscs are often consumed whole and raw or lightly cooked, their consumption results in an increased risk of illness (Goyal *et al.*, 1979; Lees, 2000).

In the environment, viruses are afforded protection from environmental sources of inactivation, such as UV rays and warm temperatures, through association with particulate matter (Grohmann and Lee, 2003). Populations of faecal coliforms tend to be higher in sediment than in the overlying water, and during rainfall, sediment is disrupted, increasing the water's turbidity (Metcalf *et al.*, 1995). Virus survival is enhanced in turbid water, and shellfish growing areas of normally good quality water are therefore at risk of contamination during periods of heavy rainfall (Grohmann and Lee, 2003).

1.4.1 Procedures employed to improve shellfish safety

1.4.1.1 Purification methods

Purification techniques are employed to reduce the microbiological load of filter-feeding shellfish. Shellfish are transferred from their normal harvesting environment to a clean supply of seawater to purge contaminating microorganisms via the natural filter-feeding process (Son and Fleet, 1980). This method is commonly practiced throughout the world to purify oysters, and is either performed in tanks in a land-based setting (depuration), or in clean waters in a natural setting (relaying) (Richards, 1988).

Water used in depuration is typically disinfected by treatment with UV. Unlike sanitizers such as chlorine or ozone, UV light leaves no residual effect to inhibit the biological processes of shellfish or shellfish taste, and is effective against both bacteria and viruses (Richards, 1988). Efficient operation of this disinfection method requires water of low to moderate turbidity, adequate flow rate, and regular cleaning of UV lamps to ensure optimal light penetration.

Whilst depuration is useful for reducing the bacteriological contamination of bivalve molluscs, viruses are removed at a slow rate. Kingsley and Richards (2003) exposed oysters to seawater contaminated with 180 plaque-forming units (PFU) per ml HAV, then performed depuration in UV-treated seawater that was changed daily for 6 weeks. In one trial, four weeks of depuration was required before HAV reached nondetectable levels in oyster tissue by tissue cell culture plaque assay, and in the second trial, HAV was still detected in oysters by reverse transcription-polymerase chain reaction (RT-PCR) 6 weeks post-contamination. The results demonstrated that the popular practice of depuration for between 36-48 h may be insufficient for removing HAV from shellfish.

Relaying is an alternative purification process that does not require a water pumping facility. Bivalve molluscs of poor microbiological quality are transferred to clean shellfish growing water, often for a longer duration than that of depuration (at least 14 days; Bird, 1994). The process may be applied if the bacterial counts in shellfish cannot be sufficiently reduced in the relatively short purification time of depuration (Richards, 1988).

Dore *et al.* (1998) reported that levels of male-specific bacteriophages, used in their study as indicators of viral contamination, were not eliminated from oysters after 4 weeks of relaying followed by depuration. Factors thought to affect the rate that contaminants are purged from oysters include oyster species, water temperature and the initial level of contamination (Dore *et al.*, 1998).

1.5 INACTIVATION OF HUMAN ENTERIC VIRUSES

Most viruses are inactivated by heating to temperatures typically used in cooking, and complete inactivation of HAV is reportedly achieved within 4 min exposure to 70°C, 30 s at 75°C, 5 s at 80°C and immediately after exposure to 85°C (Parry and Mortimer, 1984). Strong oxidizing agents, such as chlorine, ozone, and UV light in water or on surfaces are effective against viruses (Blackwell *et al.*, 1985); however, HAV is quite resistant to drying (Conaty *et al.*, 2000) and can survive in the environment for an extended period of time (Hewitt and Greening, 2004; Kingsley and Richards, 2003; Croci *et al.*, 2002; Grohmann and Lee, 2003).

Inactivation by exposure to UV, hypochlorite (1.2-1.25 ppm) or 72°C is due to conformational change of capsid proteins affecting the function of antigenic sites and receptor attachment sites. Virus inactivation almost always accompanies loss of virus attachment (Nuanualsuwan and Cliver, 2003). Therefore, in order to inactivate viruses in foods using a processing method, either the protective capsid layer must be denatured or disrupted so the virion cannot attach to a host cell, or the nucleic acid contained in the particle must be damaged to an extent preventing replication in a host cell.

1.6 NONTHERMAL PRESERVATIVE PROCESSES

It is difficult to educate a population on the health benefits of changing their eating habits from traditionally consuming raw shellfish to thoroughly cooking shellfish before consumption (Halliday *et al.*, 1991; Salamina and D'Argenio, 1998). An epidemiological survey conducted in Naples, Italy, concluded that the common consumption of shellfish by the population was not affected by the awareness of the high incidence of hepatitis A infection in the region and knowledge of its route of transmission, and that the common conditions used in shellfish cooking were frequently insufficient to inactivate viruses prior to consumption (Salamina and D'Argenio, 1998). Thus an alternative non-thermal or low-heat preservative process would be valuable to reliably improve the margin of safety associated with consumption of raw product. Due to the traditional consumption of oysters raw or minimally cooked, this process must not only ensure a microbiologically-safe food, but also provide a product that is almost identical to the raw product in organoleptic quality. To ensure that harvest water quality and good manufacturing practices are not compromised, the introduction of such a process must add to, and not replace, current standard procedures.

The application of heat has long been recognized as a process that prolongs the shelf-life of foods while improving food safety, but in some products heating can cause undesirable changes affecting product organoleptic and nutritional qualities. For example, food textures are usually altered, some vitamins are known to degrade, and vegetable tissues are often softened during thermal processing and may require application of chemicals to regain firmness (San Martin *et al.*, 2002).

Consumers are increasingly demanding food products that are fresh-tasting, nutritious and convenient. At the same time, consumer concerns about food safety have steadily increased as the incidence of reported foodborne illnesses has continued to rise. These trends have fuelled interest in non-thermal processing technologies, such as high hydrostatic pressure processing, irradiation, pulsed electric fields, and high-intensity pulsed light. In comparison to traditional thermal processing methods that often cause detrimental changes in foods, these non-thermal processing techniques offer benefits such as the potential for minimising or eliminating extensive thermal processing and chemical preservatives, and limiting unfavourable effects on food quality. Preservation of freshness and protection of flavour, appearance and nutritional value results in a high quality food product, often with extended shelf-life. For these reasons, non-thermal processing technologies offer the ability to produce foods with improved quality, increased consumer appeal and a value-added premium price. Although commercialisation of these technologies has been slow to date, the above trends plus improvements in efficiency and reductions in cost mean that the rate of adoption of non-thermal processes is likely to increase.

1.6.1 High pressure processing (HPP)

Today, high pressure pasteurisation has become a commercial reality with several high pressure-treated fruit- and vegetable-based refrigerated food products currently on the international market, including a range of juices and fruit smoothies, jams, applesauce-fruit blends, guacamole and other avocado products, tomato-based salsas and fajita meal kits containing acidified sliced capsicum and onions and heat and serve beef or chicken slices (precooked).

Additionally, ready-to-eat meat products and seafood, including oysters, are on the market in the U.S.A. and Europe (Smelt, 1998; Stewart and Cole, 2001). A batch processing system is typically used where the product is placed into a final flexible consumer package before pressurization. The packages are loaded into a basket and placed into the pressure vessel, where they are submerged in a liquid of low compressibility (typically potable water). Once loaded and closed, pressures ranging from 100 to 700 MPa are normally generated by pumping additional water into the vessel. The process is relatively energy-efficient, requiring a similar amount of energy to raise the pressure to 400 MPa, as required to heat to 30°C (Cheftel, 1995). Once the desired target pressure is achieved, no further energy is required to sustain that pressure (Farr, 1990). Unlike thermal processing, pressure is distributed instantaneously and uniformly throughout foods, ensuring a homogenous treatment regardless of the size or shape of the product (Hoover, 1993).

The treatment of foods with HPP involves compressing the water surrounding the food (Barbosa-Canovas *et al.*, 1998.). Although its compressibility is low, the volume of water is decreased by 15% at 600 MPa and 22°C (Farr, 1990). The compression of water causes a moderate increase in temperature (commonly referred to as adiabatic heat or the heat of compression), the extent of which is dependent on the initial temperature of the vessel and the rate of compression. Decompression of the vessel reverses this effect at an equivalent rate (Cheftel, 1995).

The primary advantages of HPP over thermal processing are the minimal chemical and physical effects exerted on most foods while imparting a microbial kill step. High pressure does cause a range of effects on the molecular interactions in foods. Ionic bonds and at least a proportion of hydrophobic interactions are broken or distorted by high pressure, whereas hydrogen bonds are strengthened (Hoover *et al.*, 1989), and covalent bonds are unaffected (Ledward, 1995). As a result of the pressure-induced changes to ionic bonds and hydrophobic interactions, proteins start to denature at room temperature above pressures of 100-200 MPa (Cheftel, 1995). Oligomeric structures dissociate into their subunits, monomeric structures partially unfold and denature, and proteins aggregate and gel. The conformation of proteins is altered by an increase in pressure due to irreversible changes to the secondary, tertiary, quaternary and supramolecular structures (Palou *et al.*, 1999). Denaturation may result when proteins are exposed to pressure beyond that of the individual protein-specific pressure threshold (Cheftel, 1995). The structure and function of lipids and polysaccharides are altered by HPP (Ledward, 1995); however, pressure effects on lipids are usually reversible which is often not the case for polysaccharides and proteins. Smaller molecules such as vitamin C and β -carotene are not affected by high pressure (Bull *et al.*, 2004; Cheftel, 1995). Oxidative reactions in foods and enzymatic browning in some fruits are reportedly enhanced by HPP, while partial discoloration has been reported in treated red meats (Cheftel, 1995; Ledward, 1995).

1.6.1.1 Pressure effects on microorganisms

The required pressure treatment for microbiologically safe and stable products is dependent on the target microorganism to be inactivated. Bacterial vegetative cells, yeasts, moulds and some viruses are sensitive to pressures between 200-700 MPa; bacterial spores may survive pressurization above 1000 MPa (Arroyo *et al.*, 1999; Cheftel, 1992; Sale *et al.*, 1970). Spoilage of food and/or food safety issues due to the outgrowth of bacterial spores can be controlled via complementary means such as refrigeration and acidification. Various common factors influence the pressure resistance of microorganisms, including the target microorganism and its physiological state, the intrinsic properties of the menstruum, the processing temperature, and the time and magnitude of pressure treatment (Hoover *et al.*, 1989). Considerable variation in susceptibility to high pressure has been observed among various microbial species, strains, and on microorganisms in different substrates (Patterson *et al.*, 1995). Certain foods provide microorganisms protection from inactivation or injury from high pressure. For example, milk is said to be more protective to bacteria during HPP than a buffered solution (Cheftel, 1995) or meat (Patterson *et al.*, 1995).

The critical site of pressure damage leading to inactivation of bacteria and fungi is the cytoplasmic membrane. Cell permeability is altered and ion exchange is disrupted due to crystallization of membrane phospholipids and protein denaturation (Cheftel, 1995; Yuste *et al.*, 2001). Pressure-sensitive bacteria begin to lose viability at approximately 180 MPa (Lado and Yousef, 2002). Between 200-400 MPa, irreversible changes such as cell leakage, which leads to cell death, has been demonstrated by the release of UV-absorbing material from *E. coli* (Farr, 1990; Isaacs *et al.*, 1995; Smelt, 1998).

1.6.1.2 Oyster processing

The application of HPP to whole oyster processing has been attractive for a variety of reasons. Oysters (and other shellfish) are high-value foods traditionally consumed raw throughout the world (Kingsley *et al.*, 2002). Pathogens associated with raw oysters, notably *Vibrio* spp. and HAV, are sensitive to inactivation by HPP (Calci *et al.*, 2005; Kingsley *et al.*, 2002; Styles *et al.*, 1991). The refrigerated shelf-life of harvested oysters is limited, so any extension of shelf-life without altering sensory quality is highly desirable. An extension of oyster shelf-life can be achieved by pressure treatment. Additionally, Lopez-Caballero *et al.* (2000) described pressure-treated oysters as 'slightly more voluminous with a very pleasant appearance', and reported that oysters were more appealing following treatment at chilled temperatures than at room temperature and above. Flavour may be enhanced, possibly by pressure infusion of the salty liquor within the oyster shell into the meat (Hoover *et al.*, 1989).

Adductor muscles holding oyster shells tightly closed are cleaved during high pressure treatment, ensuring convenient manual shucking of the whole oyster without the need for shucking knives (Kingsley *et al.*, 2002). This allows for higher yields, as there is both a full release of the muscle from the shell and no damage to the tissue from the shucking knife. At 275 MPa, nearly 100% of whole shell oysters are opened. Usually a hold time of 1 to 2 minutes is used. Once shucked, the oyster meat can be manually shaken off the shell and further processed in semi-rigid containers at 415 MPa for several minutes which extends the refrigerated shelf-life to three weeks (D.F. Farkas, personal communication).

Pressure treatment has been shown to speed removal of lobster meat from its surrounding shell as well.

1.6.1.3 Pressure effects on viruses

The work of Giddings *et al.* (1929) was the first documented attempt to estimate the pressure sensitivity of viruses by studying tobacco mosaic virus (TMV). TMV was found to be extremely resistant to pressure; pressurization at 920 MPa was necessary to show any measurable inactivation. Fortunately, the pressure resistance of most human and animal viruses is lower than that of TMV. Most of these viruses can be inactivated at pressures <450 MPa (Table 1.1). An exception is poliovirus, which appears to be the most pressure resistant amongst the human and animal viruses studied thus far, capable of surviving an hour at 600 MPa with only modest reductions in infectivity (Wilkinson *et al.*, 2001).

Kingsley *et al.* (2002) reported treatment with 450 MPa for 5 min at ambient temperature reduced HAV in isotonic tissue culture medium by approximately 7- \log_{10} PFU/ml. The pressure required to inactivate HAV within 5 min increased when treated in seawater of 27.4 ppt salinity (Kingsley *et al.*, 2002). Salt may act to stabilise viral capsid proteins at high pressure; but more information detailing the effect of salt on the stability of viruses during HPP will be important for future applications of HPP to shellfish products.

Table 1.1. Pressure inactivation of viruses.

Virus name	Enveloped?	Pressure (MPa)	Time (min)	Temperature (°C)	Loss in infectivity (log ₁₀)	References
Aichivirus A846/88	no	600	5	21	no reduction	Kingsley <i>et al.</i> , 2004
Coxsackievirus A9	no	500	5	21	7	Kingsley <i>et al.</i> , 2004
Coxsackievirus B5	no	600	5	21	no reduction	Kingsley <i>et al.</i> , 2004
Feline calicivirus	no	275	5	22	7	Kingsley <i>et al.</i> , 2004
Foot and mouth disease virus	no	240	120	-15	6	Oliveira <i>et al.</i> , 1999
Hepatitis A virus	no	450	5	22	7	Kingsley <i>et al.</i> , 2002
Herpes simplex virus type 1	yes	400	10	25	7	Nakagami <i>et al.</i> , 1992
Human cytomegalovirus	yes	300	10	25	4	Nakagami <i>et al.</i> , 1992
Human immunodeficiency virus	yes	350	10	25	>3	Nakagami <i>et al.</i> , 1996
Human Parechovirus-1	no	500	5	21	4	Kingsley <i>et al.</i> , 2004
Infectious bursal disease virus	no	230	120	0	5	Tian <i>et al.</i> , 2000
Murine norovirus	no	450	5	20	6.85	Kingsley <i>et al.</i> , 2007
Phage λ		400	20	22	7	Chen <i>et al.</i> , 2004
Poliovirus	no	600	60	20	<1	Wilkinson <i>et al.</i> , 2001
Rotavirus	no	300	2	25	8	Khadre and Yousef, 2002
Simian immunodeficiency virus	yes	250	60	22	5	Jurkiewicz <i>et al.</i> , 1995
Sindbis virus	no	250	480	not specified	5	Gaspar <i>et al.</i> , 1997
Vesicular stomatitis virus	yes	260	720	20	4	Silva <i>et al.</i> , 1992

Modified from Grove *et al.*, 2006.

The HAV capsid reportedly remains intact following inactivation by HPP (Kingsley *et al.*, 2002). HPP may therefore denature the capsid proteins essential for host cell attachment to initiate infection, but not release RNA from virions (Khadre and Yousef, 2002; Kingsley *et al.*, 2002). RT-PCR performed on the RNA from non-infectious virions still yielded a positive result, demonstrating its unreliability for determining the viability of pressure-treated virus.

HAV could not be recovered from strawberry puree or sliced green onions following 5 min exposure to 375 MPa, a reduction of 4.32- \log_{10} and 4.75- \log_{10} PFU, respectively (Kingsley *et al.*, 2005). Rotavirus titre was found to decline by 5- \log_{10} 50% tissue culture infective dose units (TCID₅₀)/ml within a 70-s exposure to 300 MPa in tissue culture media at 25°C, but 1- \log_{10} TCID₅₀/ml still remained after a 10-min treatment (Khadre and Yousef, 2002). Herpes simplex type 1 virus and human cytomegalovirus were inactivated by >7- \log_{10} and >4- \log_{10} after 10-min exposure to more than 400 MPa in tissue culture media at 25°C (Nakagami *et al.*, 1992). Damage to the viral envelopes prevented virions from binding to host cells and subsequently initiating infection.

A 7- \log_{10} TCID₅₀/ml culture of the norovirus surrogate, feline calicivirus (FCV), was completely inactivated (detection limit 10^{0.5} TCID₅₀) in isotonic tissue culture medium after 5 min exposure to 275 MPa or more (Kingsley *et al.*, 2002). This highlights the potential for inactivating human norovirus with HPP, but cannot be relied upon to guarantee the susceptibility of norovirus to the process. For example, HAV and poliovirus are both members of the picornavirus family, but have largely differing susceptibilities.

More recently, a murine norovirus (MNV-1) was found to possess greater stability to HPP than FCV, with a 5 min treatment at 450 MPa required for a 6.85- \log_{10} PFU reduction in titre (Kingsley *et al.*, 2007). MNV-1 has more biochemical, pathological and molecular similarities to human noroviruses than FCV (Wobus *et al.*, 2006; Cannon *et al.*, 2006), and is therefore likely to also be a better indicator of the sensitivity to HPP of human norovirus.

The extent of virus inactivation is dependent upon treatment pressure duration and temperature. Jurkiewicz *et al.* (1995) studied the pressure sensitivity of simian immunodeficiency virus (SIV), and observed a 5- \log_{10} reduction in SIV infectivity after a 1-h exposure to 250 MPa at 21.5°C. Treatments at 200 and 150 MPa required 3 and 10 h, respectively, to attain equivalent reductions of 5- \log_{10} infectious units.

A number of reports have indicated that the dissociation and denaturation of proteins and viruses by pressure is promoted by low temperatures (Bonafe *et al.*, 1998; Foguel *et al.*, 1995; Gaspar *et al.*, 1997; Kunugi and Tanaka, 2002; Tian *et al.*, 2000; Weber, 1993). The explanation for this phenomenon is that low temperatures promote the exposure of non-polar side chains to water. The non-polar interactions are more affected by pressure because they are more compressible. Oliveira *et al.* (1999) examined the combined effect of pressure and low temperature on the stability of foot-and-mouth disease virus (FMDV), an animal virus that can cause devastating losses in the meat and dairy industries. FMDV was found to be sensitive to pressure: exposure to 240 MPa for 2 h resulted in loss of infectivity of 4- \log_{10} infectious units at room temperature and

6-log₁₀ units at -15°C. Interestingly, this effect was not noted for HAV by Kingsley *et al.* (2006), who reported enhanced inactivation from high pressure treatment at 50°C compared to treatment at -10 and 20°C.

1.7 PREDICTIVE MICROBIOLOGY

HPP may have the potential to improve the microbiological safety of shellfish consumption, but its implementation, as is the case for the commercialisation of any novel food process, is reliant on obtaining systematic inactivation kinetic data of specific target microorganisms (Stewart and Cole, 2001; Lado and Yousef, 2002). This data, applicable over a wide range of conditions, forms the basis of a kinetic inactivation model.

1.7.1 Model development

In predictive microbiology, microbial growth, survival or death in a specific food may be described or predicted by a mathematical model formed from data collected from microbial responses to defined and controlled conditions. By interpolation, a model can predict the microbial response to specific conditions not actually tested in the laboratory, and be a cost-effective substitute for performing traditional shelf life and food safety studies (Ross and McMeekin, 1991).

The first consideration when designing a model is identifying its intended use (Legan *et al.*, 2002). Inactivation models predict the response of a microorganism over a defined range of a lethal process, such as heat or high pressure. Other factors, such as pH and water activity, act in combination with the lethal process to influence microbial death, and must also be identified for study within a realistic range. Three or four factors should be investigated over ranges that will allow coverage of all possible and likely responses in the intended process. Replication assists in minimising the impact of the variability in the microbial response (Legan *et al.*, 2002). To identify any curvature and to gain a true understanding of the microbial response during the treatment, 10-15 data points should be measured throughout the process (McMeekin *et al.*, 1993). This is known as a kinetic study, and is preferred to an end point measurement because more information is gained, and the failure point in the process can be identified (Stewart and Cole, 2001).

The need for more kinetic inactivation data prior to the commercialisation of HPP for pathogen reduction has already been highlighted (IFT, 2000). Thermal processing has long been used in food production, and the effect of heat on most known microorganisms and foods is known. In this case, the 'processing continuum' has provided food processors with an abundance of literature with which to base decisions regarding changes in food constituents or production regime (Legan *et al.*, 2002). For processors wishing to implement a novel non-thermal technique into food production, however, a great deal of kinetic inactivation data is required for each relevant pathogenic microorganism.

Creation of a model should involve experimentation in a homogeneous medium, for example, liquid culture media, which is less complex than the food to which validation of the model will occur (Legan *et al.*, 2002). In this way, the number of controlling factors can be limited as required. The controlling factors used to create a model, and the range over which each factor exists must be carefully chosen, as a model may only be applied within these conditions. If not applied within these specific conditions, the underlying principle for obtaining the data is lost because the action of interacting factors outside the model's determined range will be unknown. This is because currently available models are empirical, not mechanistic, and so there is no theoretical basis for extrapolation of models beyond the bounds of the data used to generate them.

1.7.2 Model validation

Validation of a predictive model is achieved by comparing data gained in a real food system with predictions made by the original model, and making appropriate modifications, where required (McMeekin *et al.*, 1993). Validation of the model is specific to each particular food. Process conditions can be adjusted according to the microbial protection afforded by the food, allowing optimised processes for a variety of foods.

1.8 PROJECT AIMS

Evaluate and compare cultivation methods for HAV to produce moderately large volumes of high titre virus to be used in challenge testing

Evaluate and compare methods for extraction and purification of HAV from oysters to ensure high percentage recovery

Develop a method to artificially inoculate live oysters with HAV, using the natural filter-feeding process of oysters

Collect HPP kinetic inactivation data for HAV suspended in buffered media, and develop a predictive inactivation model for HAV that may be validated in oysters naturally contaminated with HAV

Develop a quantitative real-time reverse transcription-PCR assay for HAV, and determine its ability to detect and accurately quantitate HAV extracted from contaminated oysters

Chapter 2: General materials and methods

All laboratory work described in this thesis was conducted at the Food Science Australia laboratory in Werribee (Melbourne), Victoria, Australia, unless otherwise specified.

2.1 GENERAL PROCEDURES

Glassware used during tissue culture protocols was acid-washed prior to autoclaving, by rinsing thoroughly with 1 M hydrochloric acid followed by 5 rinses with Milli-Q[®] water (Millipore, Australia). Acid-washed glassware and autoclavable plasticware were sterilised by autoclaving at 121°C for 25 min, and solutions were sterilised by autoclaving at 121°C for at least 20 min.

Centrifugation of volumes less than 2 ml was performed in a Sigma 1-15 microcentrifuge; volumes between 2 and 10 ml were centrifuged in a Sigma 2-5 centrifuge; and volumes greater than 10 ml were centrifuged in a Beckman J2-21 M/E centrifuge. Where centrifugal force greater than 15,000×g was required, a Beckman Coulter Optima L-90 K ultracentrifuge was used.

Weighing of substances more than 2 g was performed on a Mettler PM 6100 balance, and substances less than 2 g were weighed on an Sartorius A210 P analytical balance.

Socorex calibra 822 micropipettes were used for all micropipetting of liquids from 1 μ l to 1 ml. A Genex Delta pipette was used to transfer volumes of liquid from 1 ml to 25 ml.

Tissue culture cell lines were incubated in a Sanyo MCO-20AIC model CO₂ incubator at 37°C and 95% humidity in an atmosphere containing 5% CO₂.

2.2 PREPARATION OF SOLUTIONS

All solutions were prepared with Milli-Q[®] water and sterilised by autoclaving unless otherwise stated.

Acetone and methanol: A 1:1 (v/v) mixture of acetone (Sigma-Aldrich, U.S.A.) and methanol (BDH, Australia). This solution was not autoclaved.

Antibiotic/antimycotic solution: 100 \times solution (Sigma-Aldrich); added at 1:50 or 1:100, as specified. This solution was not autoclaved.

Anti-HAV monoclonal antibody in skim milk: A 1:2000 (v/v) dilution of mouse anti-HAV monoclonal antibody (CSL Ltd., Australia) in 1% (w/v) skim milk. This solution was not autoclaved.

Citrate/phosphate buffer: 0.05 M buffer prepared by mixing sterile 0.1 M citrate buffer (BDH, England) with sterile 0.1 M phosphate buffer (BDH, Australia) and diluting 1:1 in Milli-Q[®] water; pH 5.0.

Conjugated anti-mouse antibody: Anti-mouse antibody conjugated with horseradish peroxidase (Sigma-Aldrich), diluted 1:1000 (v/v) in PBS. This solution was not autoclaved.

Dulbecco's modification of Eagle's minimum essential medium (DMEM): 500 ml DMEM was prepared by mixing 100 ml sterile 5× DMEM (Thermo Electron Corp., Australia) with 372 ml sterile Milli-Q[®] water, 10 ml sterile 1 M HEPES (Thermo Electron Corp.), 13.5 ml sterile 7.5% sodium bicarbonate (Thermo Electron Corp.) and 4.5 ml sterile liquid L-glutamine (Thermo Electron Corp.).

Ethidium bromide (EtBr): Prepared by diluting 0.625 mg/ml EtBr (Mercury, U.S.A.) to 0.5 µg/ml in deionised water. This solution was not autoclaved.

Glycine buffer: 0.1 M glycine (Calbiochem, U.S.A.), 0.3 M NaCl (Chem-Supply, Australia); pH 9.5.

GNT: 80% (v/v) glycerol (BDH, Australia), 100 mM NaCl (Chem-Supply), 100 mM Tris (Sigma-Aldrich); pH 7.4.

Growth media: DMEM with 10% (v/v) newborn bovine serum (NBS; Thermo Electron Corp.). This solution was filter-sterilised through a 0.2 µm filter before use.

Non-ionic detergent: Igepal[®] CA-630 (Sigma-Aldrich) diluted to 10% in Milli-Q[®] water. This solution was not autoclaved.

NT buffer: 100 mM NaCl (Chem-Supply), 10 mM Tris (Sigma-Aldrich); pH 7.4.

PEG solution: 16% (w/v) polyethylene glycol (PEG) 8000 (Sigma-Aldrich), 0.525 M NaCl (Chem-Supply).

Phosphate-buffered saline (PBS): Prepared by dissolving one PBS tablet (Oxoid, Australia) in 100 ml Milli-Q[®] water; pH: 7.3.

PBS and Tween 20: Prepared by mixing PBS with 1% (v/v) Tween 20 (Sigma-Aldrich). This solution was not autoclaved.

Seawater: 30 parts per thousand (ppt) prepared unless otherwise specified, by dissolving 34 g Red Sea salt (Red Sea Fish Pharm, Israel) per litre of deionised water. Salinity was calculated using a hydrometer, thermometer, and conversion table (Bird, 1994). This solution was not autoclaved.

Skim milk: Prepared by dissolving skim milk powder (Nestle, Australia) in PBS. This solution was not autoclaved.

Sodium lauryl sulfate (SDS): Prepared by dissolving SDS (Sigma-Aldrich) in Milli-Q[®] water. This solution was not autoclaved.

Sucrose in NT: Prepared by dissolving sucrose (AJAX, Australia) in NT buffer.

Substrate solution: O-phenylaminediamine (OPD, 0.6 mg/ml; Sigma-Aldrich) and hydrogen peroxide (30% (w/v); BDH) diluted 1:2400 (v/v) in 0.05 M citrate/phosphate buffer. This solution was not autoclaved.

TBE buffer: Made to 10× stock solution and diluted to 1× prior to use. To make 10× stock solution, 10.8% (w/v) Tris (Sigma-Aldrich), 5.5% (w/v) boric acid (Calbiochem) and 3.7% (w/v) ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich) were dissolved in Milli-Q® water. This solution was not autoclaved.

Trypsin: EDTA: Pre-prepared sterile 1:250 mixture (Thermo Electron Corp.); stored at -20°C in 30 ml aliquots.

2.3 GENERAL TISSUE CELL CULTURE METHODS

2.3.1 Maintenance of tissue culture cell lines

A continuous African Green Monkey kidney cell line (BSC-1) was grown to confluence in a 25 or 75 cm² flask twice weekly, and passaged by removing growth media and then washing cells twice with sterile PBS. Cells were detached from the base of the flask by trypsinisation; where cells were incubated at 37°C for 5 min with 1 or 2 ml trypsin:EDTA (for 25 or 75 ml² flasks, respectively). Five millilitres DMEM was then added to the flask and the entire contents decanted into a sterile 10 ml centrifuge tube. The cells were pelleted by centrifugation at 500×g for 2 min, then resuspended in 4 ml DMEM. A new 25 or 75 cm² tissue culture flask was seeded with 1 ml resuspended cells, and then filled with fresh growth media. For virus quantification in 96-well microtitre plates, 1 ml of resuspended cells was diluted in 9 ml of growth media, and 0.1 ml seeded into each well. BSC-1 cells between passage numbers 59 and 79 were used in all experiments.

2.3.2 Storage of cell lines

Trypsinised and pelleted cells derived from a 25 cm² flask were resuspended in 0.9 ml DMEM and transferred to a sterile 2 ml cryotube containing 0.1 ml dimethylsulphoxide (DMSO; Sigma-Aldrich). Tubes were mixed gently by inversion and transferred to -70°C for 24 h prior to long-term storage in liquid nitrogen. Cells derived from a 75 cm² flask were distributed into four cryotubes.

Cells recovered from long term liquid nitrogen storage were thawed quickly under warm water, transferred to 5 ml DMEM slowly (dropwise), and centrifuged for 2 min at 500×g. The supernatant was then removed and the pelleted cells resuspended gently in DMEM and seeded into a 25 cm² tissue culture flask with fresh growth media.

2.3.3 Quantification of infectious hepatitis a virus

An enzyme immunoassay, modified from Borovec and Uren (1997), was used to determine infectious HAV titre. Serial 10-fold dilutions of HAV (strain HM-175, kindly donated by Prof. David Anderson, MacFarlane Burnet Institute, Australia) were made in DMEM + 2% NBS, and 0.1 ml of each dilution was transferred to 5 replicate wells containing a monolayer of BSC-1 cells (approximately 50% confluent) on a 96-well microtitre plate. In low titre and/or low volume samples, a 1:2 (v/v) or 1:4 (v/v) dilution of sample was analysed in the assay to increase the assay's detection limit.

After 7 days incubation, media was removed from wells, and cells fixed to the base of wells by addition of a 1:1 mixture of acetone and methanol (pre-cooled to 4°C). The fixative was removed after 5 min and plates allowed to dry by standing uncovered for at least 1.5 h at room temperature. One hundred microliters of a 1:2000 dilution of anti-HAV monoclonal antibody in skim milk was added to each well to detect HAV antigen. The antibody solution was removed after 1 h incubation at 37°C, and wells were washed three times with 0.1 ml PBS and Tween 20. One hundred microliters of conjugated anti-mouse antibody was added to each well and incubated for 1 h prior to another wash step. Colour was

visualised upon the addition of 0.1 ml substrate solution. The colour change was monitored visually until the reaction was stopped by addition of 0.1 ml 1 M sulphuric acid. Wells that produced visible colour to the naked eye were scored positive for HAV presence. The titre of infectious HAV was then determined by the 50% tissue culture infective dose (TCID₅₀) method, described by Reed and Muench (1938), in the units TCID₅₀/ml of undiluted sample. Detection limit of this assay is 29.5 TCID₅₀/ml when a 1:2 (v/v) dilution of sample is assayed.

Chapter 3: Evaluation and comparison of methods for high-throughput cultivation of hepatitis A virus

3.1 INTRODUCTION

For the purpose of microbiological challenge testing and vaccine production, high concentrations of microorganisms in large volumes are often required. Although HAV is cultivatable in the laboratory, it can be difficult to propagate in tissue culture cell lines, requiring lengthy incubation times (1-2 weeks) to produce a sufficiently high titre suitable for challenge testing.

The difficulty of HAV propagation is predominantly due to a large proportion (approximately 80%) of virions remaining cell-associated during infection (Nasser and Metcalf, 1987). As a result, the spread of infection throughout a tissue cell culture monolayer is relatively slow. Quantification by infectivity assay in cell culture is made more difficult by the fact that not all HAV isolates form plaques or cause cytopathic effects in cells, and those that do require long incubation of more than 10 days (Beales *et al.*, 1996; Borovec and Uren, 1997).

During cultivation of HAV, the host cell line is infected with the virus and incubated for a number of days to promote the generation of viral progeny. Virions are released from infected cells by mechanical lysis, for example, with ultrasound, by addition of a non-ionic detergent, by solvent extraction or by

sequential freezing and thawing. Additional purification of the virus stock may be required, especially for vaccine production, and this may be achieved with solvent extractions, ultracentrifugation and gel-exclusion or ion-exchange chromatography. These procedures often require specialised equipment, can be time consuming to perform and may result in loss and/or dilution of virus (Bishop *et al.*, 1994). Additionally, the use of hazardous solvents and chemicals during virus purification may be restricted in certain laboratories due to the increased occupational health and safety risks involved in carrying out the procedure.

Whilst preparation of a pure virus stock is always important, the level of purification required for production of human vaccines is much higher than that required for laboratories performing challenge studies with, for example, HPP. For example, virions tend to associate with each other in suspension, forming 'clumps', and with particulate matter in the environment, which may confer increased resistance to environmental conditions or treatment processes (Metcalf *et al.*, 1979; Landry *et al.*, 1983).

The aim of this study was to compare throughput and modify if necessary, established methods of HAV cultivation and to establish a simple and effective method for cultivating moderately large quantities of infectious HAV in high titres for challenge testing with HPP.

Two well established cultivation methods described by Bishop *et al.* (1994) and Beales *et al.* (1996) were compared in this study. The freeze-thaw method was described by Beales *et al.* to cultivate HAV stock for the development of a

cytopathic quantitative assay for HAV, and involved sequential freeze-thaw cycles to release virions from infected cells followed by a solvent extraction step to purify virions. The alternate method, described by Bishop *et al.*, involved an ultracentrifugation step through discontinuous sucrose/glycerol density gradients that could consistently yield highly purified infectious HAV particles suitable for use in vaccines and diagnostic tests.

3.2 METHODS

3.2.1 Tissue culture cells and hepatitis A virus

BSC-1 cells at 50% confluency in a 75 cm² flask were infected with approximately 10⁵ TCID₅₀ purified HAV stock. Twenty millilitres of DMEM with 2% (v/v) NBS was added to the flask and incubated for 7 days.

3.2.2 Hepatitis A virus cultivation

The cultivation method described by Bishop *et al.* (1994) was initially investigated, with 75 cm² flasks used for cell growth instead of the 6000 cm² cell factories described in the original protocol. Following the 7-day incubation described above, growth media was decanted from the flask and infected cells were washed twice with 2 ml PBS. Cells were trypsinised with 2 ml trypsin: EDTA and incubated for 5 min at 37°C. Trypsin was then inhibited by the addition of 5 ml DMEM with 5% (v/v) NBS. Cells were decanted into a sterile centrifuge tube (Nunc, Denmark) and pelleted by centrifugation at 500×g for 5 min. The cell pellet was resuspended in 5 ml DMEM and centrifugation repeated.

The final cell pellet was resuspended in 5 ml NT buffer, and cells lysed by addition of 10% non-ionic detergent to a final concentration of 1% (v/v). Nuclei were removed by centrifugation at 500×g for 5 min, and the cytoplasmic lysate, divided in 1 ml aliquots, was clarified by centrifugation in a microcentrifuge at 13,000×g for 2 min. Following centrifugation, 20% (w/v) SDS was added to a final concentration of 2% (w/v).

A discontinuous sucrose/glycerol gradient was prepared in a heat sealable centrifuge tube (Beckman, U.S.A.) by sequential layering of: 0.5 ml GNT buffer; 1.8 ml 30% (w/v) sucrose in NT buffer; 1.8 ml 20% (w/v) sucrose in NT buffer; and 1.8 ml 10% (w/v) sucrose in NT buffer containing 1% (w/v) SDS. Six millilitres of crude lysate was layered on top of the gradient, and samples were centrifuged at 170,000×g for 6 h at 18°C. The majority of virions were expected to be located in the GNT layer (containing glycerol) following centrifugation, although a small virus pellet was visible on the base of the tube. Five hundred microlitres of liquid was removed from the base of the tube by micropipette, and the remaining liquid decanted. The 0.5 ml supernatant plus an additional 1.5 ml NT buffer was added back into the tube to resuspend the pellet, and samples were stored at -70°C in 1 ml aliquots.

The freeze-thaw method described by Beales *et al.* (1996) was chosen as a relatively simple method involving minimal use of chemicals and purification steps. After cells were exposed to virus for 1 week as described in 3.2.1, growth media from the flask was decanted into a sterile centrifuge tube (Nalgene, U.S.A.), and the infected cell monolayer trypsinised with 2 ml trypsin: EDTA and

combined with the decanted media. The combined stock of infected cells and growth media underwent three freeze-thaw cycles, cycling between -70°C and room temperature, prior to centrifugation at $12,100\times g$ for 10 min at 4°C . Virus-containing supernatant fluid was extracted with an equal volume of chloroform or dichloromethane and centrifuged at $483\times g$ for 15 min at 4°C . Chloroform was initially used in the extraction step, and was later replaced with dichloromethane due to the lower rating on the latter's MSDS for chronic health effects resulting from exposure. The virus-containing aqueous layer was removed and stored at -70°C in 20 ml aliquots.

The infectious titre of viruses cultivated by each method was quantified as previously described in chapter 2, section 2.3.3.

3.3 RESULTS

The titre of HAV cultivated by the freeze-thaw method was higher than that achieved with the Bishop *et al.* method, as can be seen in Figure 3.1. Using chloroform for the solvent extraction, an average 1.1×10^5 TCID₅₀/ml was cultivated with the freeze-thaw method, and an average 4.6×10^5 TCID₅₀/ml was cultivated by the same method using dichloromethane. These titres are approximately 2-log_{10} TCID₅₀/ml greater than that cultivated by the Bishop *et al.* method (average 2.5×10^3 TCID₅₀/ml).

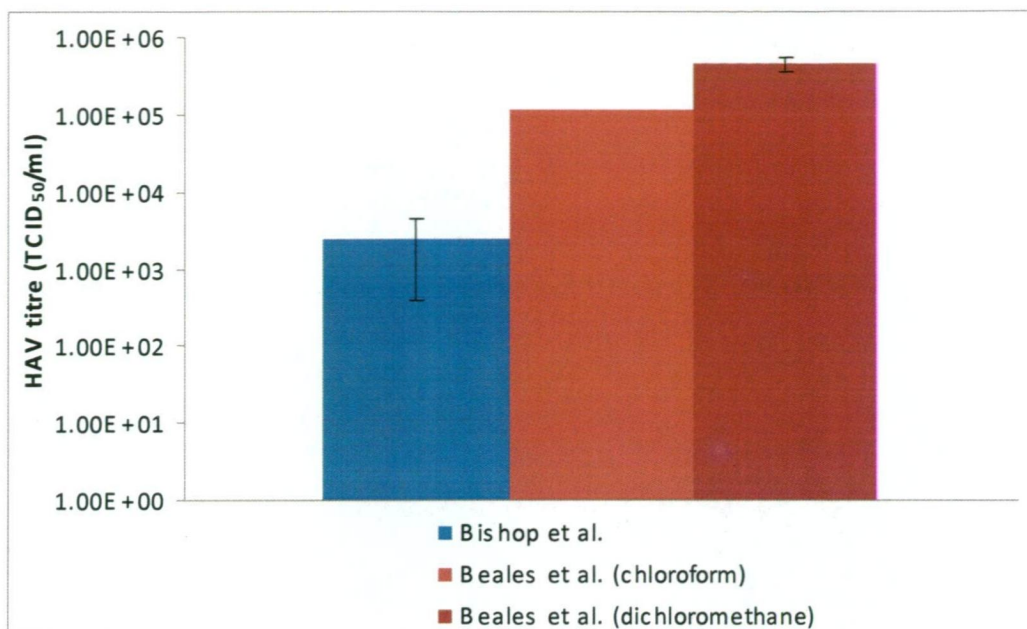


Figure 3.1. Hepatitis A virus (HAV) titre cultivated by the Bishop *et al.* method and the freeze-thaw method (Beales *et al.*, 1996), with a solvent extraction step using either chloroform or dichloromethane. Columns indicate the average of at least triplicate cultivations, except for the freeze-thaw method (chloroform extraction; performed in duplicate). Error bars represent standard deviation between at least triplicate samples.

The volume of HAV cultivated by the freeze-thaw method was larger than that cultivated by the Bishop *et al.* method. The total volume of HAV cultivated by the freeze-thaw method was equivalent to the volume of growth media in each flask (approximately 20 ml per 75 cm² flask), as infected tissue culture cells were disrupted during freeze-thaw cycles to release virions into growth media collected from infected cell culture flasks. The volume of HAV cultivated by the Bishop *et al.* method was approximately 10-fold lower (Table 3.1), with 2 ml purified HAV collected after the ultracentrifugation step.

The sucrose/glycerol gradient used in the Bishop *et al.* method was important in cushioning virions during ultracentrifugation, but was instead disrupted during the cycle because of the unavailability of swing-out rotors in the laboratory in which HAV was cultivated. A fixed angle rotor was used instead, and as a result, the virus-containing glycerol layer at the base of the tube was not visible at the end of the ultracentrifugation cycle; instead a small viral pellet formed near the base of the tube and the pellet was resuspended in a mix of 1.5 ml NT buffer and 0.5 ml supernatant from the base of the tube, as described in section 3.2.2.

Table 3.1. Comparison of hepatitis A virus (HAV) cultivation methods by titre and volume. Titre and volume are averages of triplicate cultivation procedures, except for the freeze-thaw method with a chloroform extraction (in duplicate). *SD*: Standard deviation.

Method of cultivation		Cultivated HAV	
		Titre (TCID ₅₀ /ml) (<i>SD</i>)	Volume per flask (ml)
Bishop <i>et al.</i>		2.5×10 ³ (2.1×10 ³)	2.0
Beales <i>et al.</i>	Chloroform extraction	1.1×10 ⁵ (6.7×10 ⁴)	20
	Dichloromethane extraction	4.6×10 ⁵ (1.1×10 ⁵)	20

The Bishop *et al.* method required careful preparation of balanced heat sealed tubes for ultracentrifugation of samples, which was a time consuming task, and the entire method took >8 h to complete. Furthermore, the maximum sample volume that could be loaded into each tube for ultracentrifugation was low (6 ml). A modification was subsequently made to eliminate ultracentrifugation. After addition of 20% (w/v) SDS to the clarified lysate, virions were precipitated from the supernatant with an equal volume of PEG solution. Samples were mixed well and incubated on ice for 1 h, then centrifuged at 10,000×g for 5 min at 4°C. Pellets were resuspended in 2.5 ml PBS and filter sterilised through a 0.2 µm syringe membrane (Pall Corp., U.S.A.) prior to quantification. PEG is commonly used to isolate and concentrate viruses from a number of environmental and food samples (Lewis and Metcalf, 1988; Jaykus *et al.*, 1996; Kingsley and Richards, 2001), and by replacing ultracentrifugation with a PEG precipitation step, HAV virions could be isolated and concentrated in a considerably shorter time.

HAV titre cultivated using the modified Bishop *et al.* method yielded a substantially higher titre than that cultivated from the original method (Figure 3.2). It was also effective in reducing the length of the cultivation protocol by approximately 6 h. However, the volume of cultivated HAV did not increase substantially (final volume of cultivated HAV was 2.5 ml). Furthermore, SDS used in the protocol was carried over to the purified virus sample and had a mild toxic effect on cells infected with 1:2 or 1:10 dilutions of HAV during infectivity assay. Because colour was visualised in wells infected with high dilutions of virus, but not in wells infected with lower dilutions, it was clear the toxicity could be diluted out.

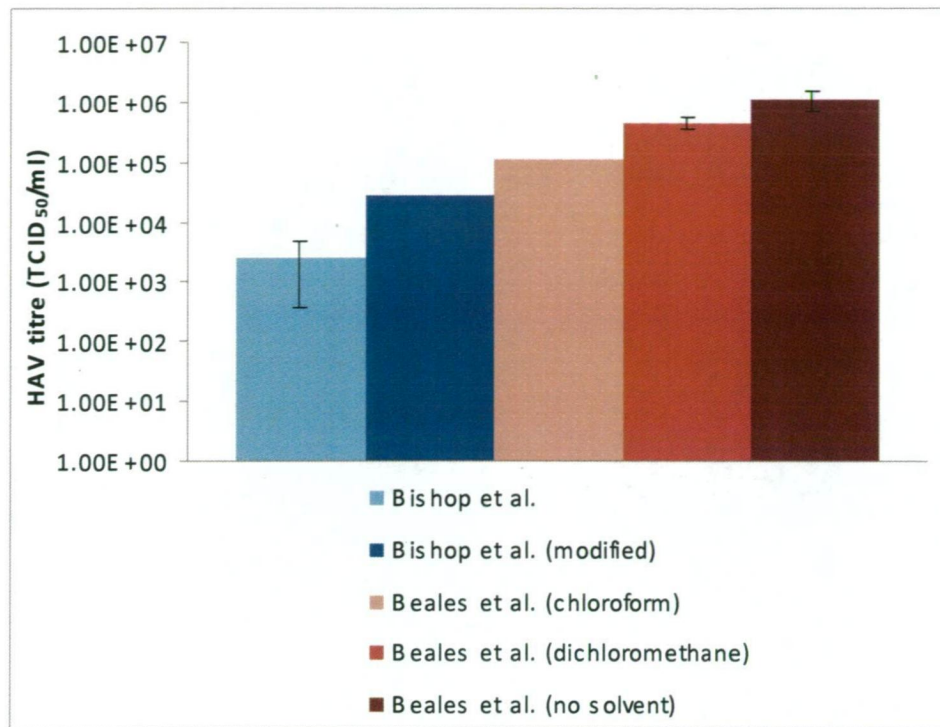


Figure 3.2. Hepatitis A virus (HAV) titres cultivated by the original and modified Bishop *et al.* methods, and the freeze-thaw method (Beales *et al.*, 1996), with either a chloroform, dichloromethane, or no extraction step. Columns indicate the average of at least triplicate cultivations, except for the freeze-thaw method (chloroform extraction; in duplicate) and the modified Bishop *et al.* method (single cultivation). Error bars represent standard deviation between at least triplicate samples.

Evidence of cell toxicity was also visible in tissue culture cells during infectivity assay when infected with the neat or 1:2 dilutions of HAV cultivated by the freeze-thaw method. Cell toxicity was characterised by the lack of colour generation in wells (false negative) and detachment of cells off the base of the 96-well plate due to cell death. To determine if this observation was due to residual solvent being carried over during HAV isolation and purification, the solvent extraction step was omitted from the protocol. After the sequential freeze-thaw cycles, samples were centrifuged as previously described, and 20 ml aliquots of virus-containing supernatant were immediately stored at -70°C .

The omission of the solvent extraction step from the freeze-thaw method improved the titres of cultivated HAV (Figure 3.2) without affecting the volume cultivated. Furthermore, the toxicity observed previously in infected cells was no longer observed, and colour formed in wells containing a 1:2 dilution of cultivated HAV was clearly visible. These results indicated the solvent extraction step was not necessary in this protocol, and its omission could ensure a more accurate quantification of HAV. Furthermore, the length of the cultivation protocol was reduced, and no solvents were required to complete the procedure.

3.4 DISCUSSION

The titres of infectious HAV cultivated by Bishop *et al.* varied between 4×10^8 and 9×10^9 radioimmunofocus-forming units (RIFU) per millilitre in the 2 ml stock purified, which could then be diluted for use as required. In the present study, however, the titres of purified HAV derived from this method were too low for additional dilution or for use in challenge studies, probably because of the use of single 75 cm² tissue culture flasks during cultivation, as opposed to the 6000 cm² cell factories described in the original method.

PEG is a water-soluble synthetic polymer commonly used to precipitate viruses from environmental samples (Lewis and Metcalf, 1988; Kingsley and Richards, 2001). It was applied in this study to replace ultracentrifugation; reducing the length of the protocol and providing an opportunity to process multiple samples and increase throughput. As a result, the titre of cultivated HAV was improved in the single trial performed. Toxicity not observed in cells during quantification of HAV using the original Bishop *et al.* method was observed in cells infected with HAV cultivated by the modified method. This correlated with a 'foamy' appearance of the virus stock upon inversion, and may indicate that SDS remained within the sample during PEG precipitation, where it had previously been removed through the sucrose/glycerol gradient during ultracentrifugation.

The cultivation and use of highly purified HAV is critical in human vaccine production, as impurities or cell debris may cause unknown side effects in vaccinated hosts. As shown in this study, cultivation of highly purified virus stock may involve an isolation and purification regime involving several hours of

time consuming and delicate centrifugation steps, or the preparation and use of chemicals and solvents that may be hazardous. It is important to obtain a pure virus stock free from as many impurities as possible, but the level of purity for virus stock used in challenge testing or process verification need not be the same as that for vaccine production. Furthermore, several authors have commented that highly purified, monodispersed virions are not indicative of the enteric viruses encountered in the environment or in contaminated foods (Eyles, 1981; Cannon *et al.*, 2006). Rather, virions tend to form aggregates and become associated with particulates, negating the need for exhaustive purification methods in the cultivation of virus stock for challenge study.

Solvent extractions are often used during isolation and purification of viruses from food and environmental samples to assist the separation and purification of virions from solid matter (Legeay *et al.*, 2000). In a virus cultivation procedure the solvent extraction may assist in the separation of virions from tissue culture cell debris; but in this case omission of the solvent extraction ensured a consistently higher titre of virus was cultivated than that achieved with the solvent extraction included. This may have been due to some carry-over of solvent into the virus stock, which may have diluted the stock by a small amount, and at the same time resulted in toxic effects in infected tissue culture cells. Challenge testing in food microbiology is frequently performed in artificially inoculated food samples where certain food components may cause detrimental effects on cell culture, so the omission of the solvent extraction ensures any toxic effects seen in cells after performing treatments on the virus stock within an inoculated food matrix is not a result of a toxic substance within the virus stock itself.

The use of solvents is discouraged in the laboratory where this work was performed due to health and safety issues. Elimination of the solvent extraction increased HAV titre and improved the readability of quantitative infectivity assays. Variation in HAV titres cultivated by the freeze-thaw method remained low, with titres ranging between 7.2×10^5 and 1.6×10^6 TCID₅₀/ml in the four trials performed. This method is therefore suitable for cultivating a moderately large amount of HAV suitable for challenge testing in the laboratory and for use in quantitative infectivity assays.

Chapter 4: Development of a method for extraction and purification of hepatitis A virus from contaminated oysters

4.1 INTRODUCTION

The accumulation of human enteric viruses from the surrounding water by filter feeding shellfish has long been a public health problem (Murphy *et al.*, 1979; Halliday *et al.*, 1996). High rainfall events may lead to a discharge of wastewater, including human faecal effluent into waterways without prior treatment, or result in run-off from farmland or recreational areas entering waterways (Murphy *et al.*, 1979). Filter-feeding shellfish such as oysters are cultivated in waterways and estuaries and may be at risk of accumulating human enteric viruses such as HAV should such discharges occur. Shellfish (Europe) or their harvest waters (Australia and U.S.A.) are routinely sampled for faecal coliform bacteria to indicate the possible presence of faecal contamination and pathogenic bacteria, but these tests are unreliable indicators of the presence of enteric viruses. For example, HAV can survive in shellfish or bound to sediment long after faecal coliform counts in the surrounding waters have returned to a level approved for shellfish harvesting (Grohmann *et al.*, 1981; Kingsley and Richards, 2003).

A number of methods to recover HAV from oyster tissue have been described in the literature by several research groups and their protocols have varied

depending on the desired downstream applications (Lewis and Metcalf, 1988; Jaykus *et al.*, 1996; Kingsley and Richards, 2001 and 2003; Mullendore *et al.*, 2001). Cell culture inhibitors that hinder the growth of or are toxic to cell lines must be removed from food samples prior to analysis of viruses in an infectivity assay, and viral genetic material should be purified free from PCR inhibitors to ensure sensitive detection by molecular techniques such as reverse transcription (RT) PCR.

Methods to extract viruses from shellfish frequently include at least one solvent extraction, usually with chloroform or trichlorofluoromethane (Freon), to assist in the purification of virions from shellfish tissue (Lees *et al.*, 1994; Dix and Jaykus, 1998; Croci *et al.*, 2000; Mullendore *et al.*, 2001). However, environmental concerns regarding the use of Freon mean it is no longer manufactured. Virus extraction methods that include multiple PEG precipitation steps and pH adjustments to improve recovery have been described, and tend to be lengthy and laborious methods that may or may not offer high virus recovery. A balance is desired between a method that can recover a high percentage of the viruses present and one that is rapid and reliable to perform, incorporating a minimal amount of dangerous chemical use.

In this chapter, methods for the recovery of HAV from spiked oyster meat are evaluated and compared. A method capable of recovering a high percentage of HAV from artificially inoculated oysters will be selected and modified if necessary, to be applied in Chapter 6 to recover low titres of infectious HAV from oyster meat post-high pressure treatment.

Kingsley and Richards (2001) developed the glycine-PEG-Tri reagent-poly(dT) extraction (GPTT) method, a method to detect HAV in shellfish by RT-PCR, which could be completed within one work day, did not involve the use of solvents, and which allowed detection of a low amount of HAV RNA in a spiked oyster sample. The same authors described a simple method to extract infectious HAV from oysters for analysis in tissue cell culture in 2003. This method did not involve multiple purification steps, did not involve solvent use, and did not contain a concentration step to increase sensitivity of the method. The method was quick to perform, allowing high throughput of samples, which is important for use in challenge studies.

These two methods were chosen for investigation and modification in this study, to ensure appropriateness of use in later challenge testing studies. Methods described by other researchers were also considered, but not included in this investigation due to their use of solvents in the method or because the method took longer than one working day to complete (Lees *et al.*, 1994; Dix and Jaykus, 1998; Croci *et al.*, 2000; Mullendore *et al.*, 2001).

4.2 METHODS

4.2.1 Tissue culture cells and hepatitis A virus

Stocks of HAV were prepared using the freeze-thaw method previously described in Chapter 3. Aliquots of virus were stored at -70°C .

4.2.2 PEG precipitation method

The GPTT method was described by Kingsley and Richards (2001) to purify virus from oyster tissue prior to extraction and purification of viral RNA for analysis with RT-PCR. Only the virus purification portion of the method was investigated, as follows:

Oyster homogenate was prepared by shucking the meat from commercially grown and harvested Pacific oysters (*Crassostrea gigas*) into a sterile blender bag, and homogenising with a stab mixer (model SM 8; Tiffany, Melbourne, Australia) for 30 s or until a smooth paste was formed.

The homogenate was diluted 1:10 (w/v) in glycine buffer, mixed by shaking for 30 s then transferred in 9 ml aliquots to sterile 40 ml centrifuge tubes (Nalgene, U.S.A.). Serial 10-fold dilutions of HAV were made in PBS from 1:1 to 1:10,000, and 1 ml from each dilution was transferred to a centrifuge tube containing the oyster homogenate and mixed by inversion. Samples were centrifuged at $15,000\times g$ for 1 h at 4°C to pellet debris. Supernatant fluids were then decanted into sterile centrifuge tubes, and HAV virions precipitated by adding an equal volume of PEG. Tubes were mixed by inversion and incubated on ice for 1 h, then centrifuged at $15,000\times g$ for 10 min at 4°C . Virus-containing

pellets were resuspended in 3 ml PBS, filter sterilised through 5, 0.45 and 0.2 μm pore size membranes to sequentially remove particulate matter, and stored at -70°C prior to analysis.

4.2.3 Crude extraction

The 'crude extraction' method was modified slightly from the method described by Kingsley and Richards (2003). Pacific oyster homogenate was diluted 1:10 (w/v) in glycine buffer, mixed well and 18 ml aliquots were transferred to 40 ml centrifuge tubes. Two stocks of HAV were diluted 1:100 (v/v) in PBS, and 2 ml of each neat and 1:100 dilution were transferred to centrifuge tubes containing homogenate. Samples were mixed vigorously for 15 s, incubated at room temperature for 10 min, then centrifuged at $15,000\times g$ for 1 h at 4°C . The pH of the virus-containing supernatant was lowered to pH 7.0 (± 0.3 pH units) using 1 M HCl, and 5 ml was removed and mixed with a 1:100 dilution of antibiotic/antimycotic solution prior to analysis.

4.2.4 Quantitative analysis

The detection limit of the infectivity assay was 148 TCID₅₀/ml initially, due to a 1:10 (v/v) dilution of purified sample being the lowest dilution to be assayed. In the samples that contained sufficient volume, a 1:2 (v/v) dilution of purified sample was analysed, increasing the assay's detection limit to 29.5 TCID₅₀/ml.

HAV recovery data gathered from each method of purification were analysed for statistical significance in Microsoft® Excel, using the Data Analysis tool.

4.3 RESULTS

4.3.1 Original PEG precipitation method

An average of 12.6% of spiked infectious HAV was recovered from oyster homogenate by the original PEG precipitation method (Table 4.1). Purified virions were pelleted alongside co-precipitated proteinaceous material from the oyster tissue, resulting in a large dark pellet following the final centrifugation. Filter sterilisation of the sample eliminated much of the particulate matter, and prevented visible contamination of the tissue culture cells, but was difficult to perform due to the co-purified material clogging up the membranes. Filter changes overcame this problem, but caused considerable loss of sample, resulting in purification of a low volume of virus.

Table 4.1. Recovery of hepatitis A virus (HAV) from artificially inoculated oyster homogenate, after extraction by the original PEG precipitation method. Titres listed are the average of triplicate infectivity assays.

SD: Standard deviation.

Initial titre		Recovered HAV	
TCID ₅₀ /ml	<i>SD</i>	TCID ₅₀ /ml	%, (<i>SD</i>)
1.52x10 ⁴	8.62x10 ³	8.73x10 ²	5.75 (0.87)
1.34x10 ⁵	1.15x10 ⁵	2.28x10 ⁴	17.0 (12.6)
1.24x10 ⁶	9.92x10 ⁵	1.85x10 ⁵	14.9 (13.8)
		Ave. recovery	12.6 (5.98)

4.3.2 Further modifications

Later, the PEG precipitation method was modified to improve recovery. Oyster homogenate was diluted 1:10 (w/v) in glycine buffer, mixed by inversion for 30 s, and 18 ml aliquots were transferred into sterile 40 ml centrifuge tubes. Two stocks of HAV, cultivated separately, were diluted to 1:100 (v/v) in PBS. Two millilitres of the 1:1 (10^6 TCID₅₀/ml) and the 1:100 (10^4 TCID₅₀/ml) dilutions were transferred to the tubes containing homogenate. Samples were mixed vigorously for 15 s and incubated at room temperature for 10 min prior to centrifugation at 15,000×g for 1 h at 4°C. Supernatant fluids were decanted into sterile centrifuge tubes, and virions precipitated by addition of an equal volume of PEG solution. Tubes were mixed vigorously, incubated on ice for 2 h, and centrifuged at 15,000×g for 10 min at 4°C. Pellets were resuspended in 5 ml PBS and filter-sterilised through 5 and 0.2 µm pore size membranes or treated with 1:100 dilution of antibiotic/antimycotic solution to remove contamination.

Antibiotic/antimycotic treatment of recovered samples lowered the variation of HAV titre between replicates considerably, compared to syringe filtration. Furthermore, the average recovery of HAV titre was significantly increased from samples inoculated with 10^4 TCID₅₀/ml HAV ($p < 0.05$), from 5.75% in the initial trials to 39.9% in the modified trials (Figure 4.1a). For syringe-filtered samples, recovered titres observed at both levels of inoculum were variable, and no significant improvement was observed as a result of the modifications made ($p > 0.05$). For example, 18.5% and 27.1% of the initial HAV inoculum was recovered from oyster homogenate inoculated with 10^6 and 10^4 TCID₅₀/ml, respectively (Figure 4.1b), compared with 14.9% and 5.75%, respectively, in the

initial trials. Particulate matter still caused difficulty during filter sterilisation, and probably contributed to the variability of recovered titres. Interestingly, the recovery of HAV from samples inoculated with 10^6 TCID₅₀/ml HAV, independent of sample treatment, was not improved significantly from the original method ($p>0.05$) (Figure 4.1a).

Later, in an effort to further improve HAV recovery, a solvent extraction step was introduced in the method. An equal volume of dichloromethane was added to the virus-containing supernatant fluid following the initial 1 h centrifugation step. The sample was mixed vigorously, and then incubated for approximately 5 min on ice for separation of phases. The virus-containing aqueous phase was removed with a pipette prior to a 2 h PEG precipitation. Finally, purified virus was divided in three, for syringe filtration through 5 and 0.2 μ m membranes, antibiotic/antimycotic treatment or left untreated prior to quantitative analysis.

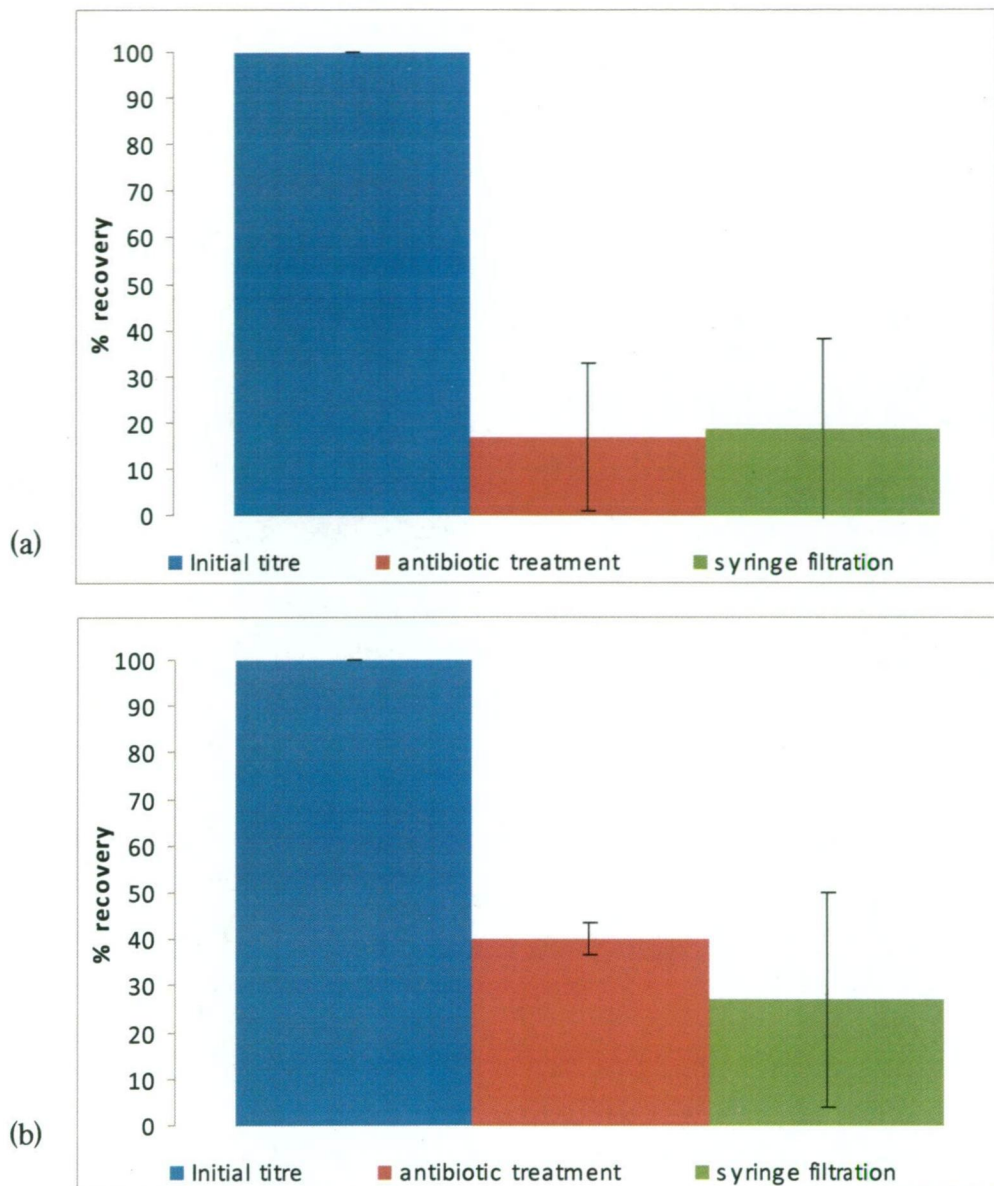


Figure 4.1. Average recovery (%) of infectious hepatitis A virus (HAV) from artificially inoculated oyster homogenate after extraction by the modified PEG precipitation method, incorporating either antibiotic treatment or syringe filtration of purified HAV. Average initial titre of HAV in (a) was 1.9×10^6 TCID₅₀/ml, and in (b) was 1.2×10^4 TCID₅₀/ml; recorded as 100% on corresponding graphs. Samples are the average of duplicate extractions, quantified by infectivity assay in triplicate, except syringe-filtered sample in (a), which is the result of one extraction assayed in triplicate.

An average of 34.1% of the HAV inoculum was recovered by the modified method incorporating antibiotic/antimycotic treatment (Figure 4.2). With no sterilisation treatment prior to infectious assay, an average 30.8% HAV was recovered, but during the infectivity assay visible contamination was introduced into the wells of the lowest dilutions (1:2 and 1:10), lowering the sensitivity of the assay because generation of fluorescence in these wells was prevented. This reiterates the requirement for sample treatment prior to cell culture; however syringe filtration was not a suitable method. Recovery dropped to 3.1% in syringe-filtered samples, although this is more likely indicative of the variable nature of syringe filtration than of solvent extraction.

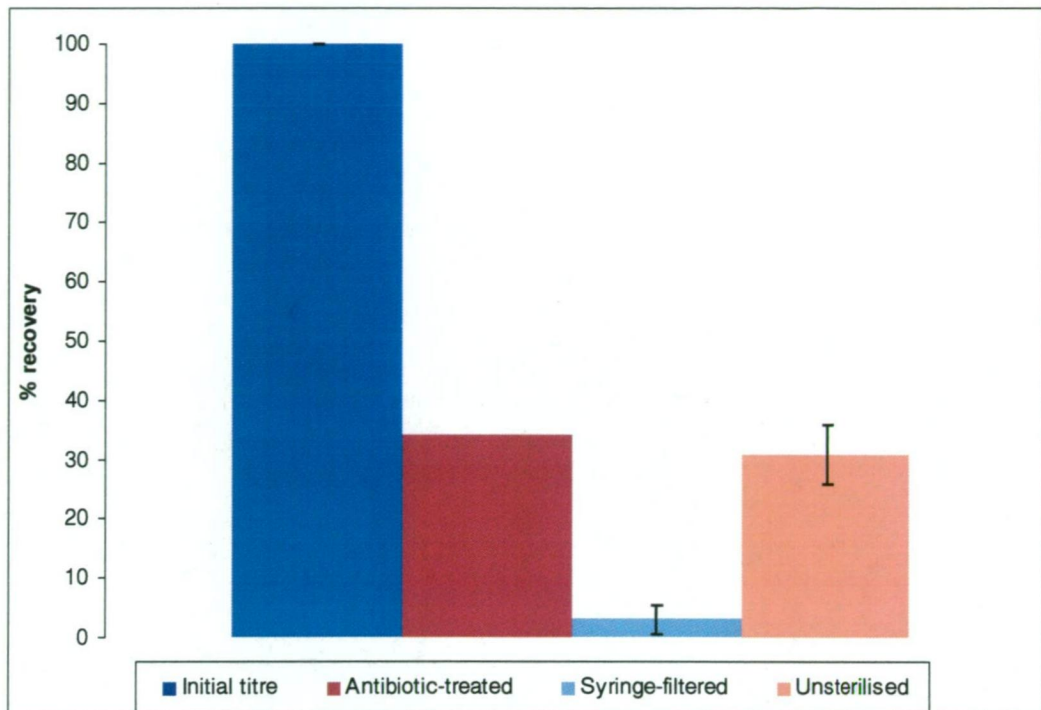


Figure 4.2. Average recovery (%) of infectious hepatitis A virus (HAV) from artificially inoculated oyster homogenate, after extraction by the modified PEG precipitation method with a solvent extraction step. Average initial titre of HAV was 3.2×10^5 TCID₅₀/ml, and is recorded as 100% in the graph. Sample recoveries are calculated from the average of triplicate infectivity assays, except for the unsterilised sample (in duplicate). Error bars represent standard deviation between at least triplicate samples.

4.3.3 Crude extraction

Relatively high recoveries of HAV were obtained from oyster homogenate using the crude extraction method (Figure 4.3). For samples inoculated with 1.9×10^6 TCID₅₀/ml, recovery averaged 44%, and was significantly higher than the PEG precipitation method incorporating antibiotic/antimycotic treatment ($p < 0.05$), but no statistical significance was observed from the syringe-filtered samples ($p > 0.05$). For samples inoculated with 1.2×10^4 TCID₅₀/ml, recoveries were significantly higher than those obtained in all modifications of the PEG precipitation method ($p < 0.05$), despite there being no concentration step in the method. The average recovery of this inoculum was particularly high in this case (119%), resulting from a recovery of 202% spiked HAV in a single trial.

The crude extraction method is simpler to perform and is completed in less time than the PEG precipitation method. Of course, the time taken to complete each method varied depending on the number of samples processed and equipment used, but for the processing of one sample by each method, a time saving of at least 2 h was achieved. Starting from the 1 h centrifugation step, the crude extraction method took approximately 1.5 h per sample to complete, compared with approximately 3.5 to 4 h to complete the PEG precipitation method with the modifications described, depending on whether samples are treated with antibiotic-antimycotic solution. Addition of a solvent extraction step increased the length of the procedure by approximately 1.25 h. These time savings were of higher importance when sample number was increased, with a larger number of samples able to be processed within a work day using the crude extraction method.

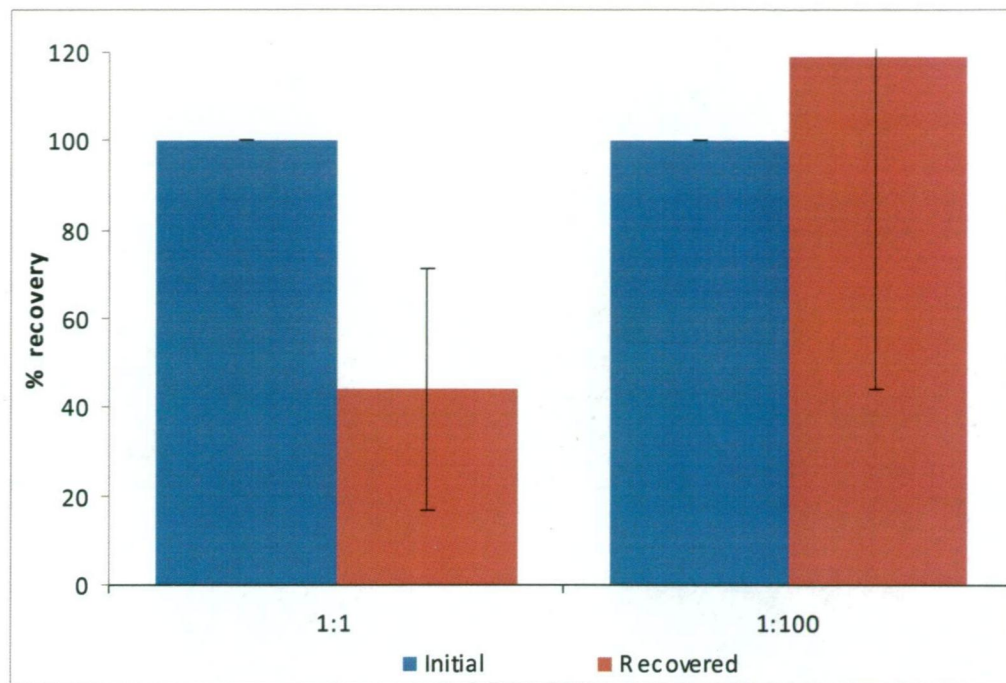


Figure 4.3. Recovery of hepatitis A virus (HAV) from artificially inoculated oyster homogenate after extraction by the crude extraction technique. Average initial titre of 1:1 HAV was 1.9×10^6 TCID₅₀/ml and 1.2×10^4 TCID₅₀/ml for 1:100 HAV, and each is recorded as 100% on the graph. Each sample is the average of duplicate extractions, quantified by infectivity assay in triplicate.

4.4 DISCUSSION

The results of this study demonstrate the considerable variability that can be encountered when recovering viruses from complex food samples. This variability has also been observed within studies and between studies reported in the literature recovering viruses from artificially inoculated oyster samples. For example, Lewis and Metcalf (1988) reported recovery of $97\% \pm 20\%$ using a PEG precipitation method, and $49\% \pm 1\%$ using an organic flocculation method. Jaykus *et al.* (1996) recovered between 5 and 10% of infectious HAV using a purification method that involved Freon extraction, PEG precipitation, and Pro-cipitateTM adsorption-elution-precipitation technique.

In the current study, the mean recovery of HAV from artificially inoculated oyster homogenate using the crude extraction method was $>40\%$, and as high as 119%. Recoveries of more than 100% of infectious virus from contaminated shellfish have been reported on several occasions by other researchers (Landry *et al.*, 1982; Lewis and Metcalf, 1988), and may be explained by the formation and dispersion of virus clumps within the sample. Virus stock was not filtered to disperse clumps during inoculation of oyster homogenate, as the clumping and aggregation of virions is more representative of viruses in their natural state (Eyles, 1981; Cannon *et al.*, 2006).

Kingsley and Richards (2003) recovered 34,000 PFU HAV using their crude infectious HAV extraction method from a live oyster initially exposed to 90,000 PFU. Assuming that 100% of the inoculum was taken up by the oyster, this represents recovery of 37% of the initial contaminating HAV. The crude

extraction method described in this chapter was modified slightly from that described by Kingsley and Richards, and recovered an average of 44% and 119% of the initial 1.9×10^6 TCID₅₀/ml and 1.2×10^4 TCID₅₀/ml HAV inoculums, respectively. However, it must be said that achieving 100% accumulation of virus by a filter-feeding oyster in the laboratory is often difficult to achieve using a small-scale recirculating contamination system, as setup in our laboratory and described in chapter 6. In such a case, the actual recovery from contaminated oysters may be higher than reported, and within the range reported in this chapter.

Kingsley and Richards (2003) reported the presence of plaque assay inhibitors and cytotoxic substances in the HAV extracts, which inhibited the plaque assay at low dilutions. In the present study, quantification of infectious HAV was achieved using an enzyme immunoassay, which does not require plaque formation for positive quantification. Some toxicity-induced morphological changes to cells were observed in those cells infected with 1:2 and 1:10 dilutions of extracted HAV, but these changes did not affect antibody binding to HAV capsid proteins within wells during immunoassay, or the subsequent colour production from the conjugated antibody, indicating the compatibility of the two methods for this analysis.

Recovery of HAV using the crude extraction technique improved with a lower titre of spiked HAV. Whilst an average of 44% of neat spiked HAV was recovered from oyster homogenate, an average of 119% of 1:100 diluted HAV was recovered from spiked homogenate, suggesting there may be an upper limit to the amount of virus able to be recovered using this purification method. Jaykus

et al. (1996) described improved recovery efficiency of both HAV and poliovirus from spiked oyster homogenate as the input titre decreased to 10^2 PFU. Landry *et al.* (1982) also reported increased poliovirus recovery with decreasing input titre. This is an important observation because the contamination of shellfish with human viruses probably occurs most frequently at a relatively low level. Whilst the specific infectious dose of these viruses remains unknown, it is believed to be <100 virions for human norovirus and HAV (Greening, 2006), and therefore the need for efficient and sensitive recovery and accurate quantification of contaminating virus is important.

The modifications made to the PEG precipitation procedure improved the recovery of HAV, although not substantially in the single trial in which a solvent extraction step was added. A number of methods have been described for the purification of viruses from shellfish, which include a solvent extraction step prior to quantification by tissue culture infectivity assays (Dix and Jaykus, 1998; Mullendore *et al.*, 2001) or detection by RT-PCR (Atmar *et al.*, 1995; Jaykus *et al.*, 1996; Croci *et al.*, 2000). An extraction and purification method that does not include the use of solvent is preferred in the current study, but was included in a modification of the PEG precipitation method for comparison.

Inoculating viral extracts into a tissue culture assay without prior treatment is certain to introduce bacterial or mould contamination, which in turn reduces the reliability of the assay. Antibiotic/antimycotic treatment of the purified samples eliminates contamination without loss of sample, and has been used for this purpose by other researchers (Landry *et al.*, 1982; Lewis and Metcalf, 1988; Lees

et al., 1994 and Lees, 2000). The treatment is a simple and rapid step for elimination of contaminants in the virus sample, shortening the length of the purification without inducing a cytotoxic or inhibitory effect on tissue culture cells or infectious virus, and the final volume of purified virus was higher than that following syringe filtration.

Co-precipitated solids in the purified virus sample generally made syringe filtration difficult prior to tissue culture quantification, and performing membrane changes during filtration resulted in a loss of sample volume. Furthermore, virus recovery following membrane filtration was generally lower and more variable than recovery following antibiotic/antimycotic treatment. Low protein-binding filters were used in the study to reduce the retention of virions within the filter membranes, but if associated with particulates of sufficient diameter, passage of virions through filter membranes may still have been hampered. Similarly, Sobsey *et al.* (1978) reported considerable retention of viruses filtered through a 0.2 μ m filter following extraction from oyster tissue; a conceivable occurrence due to association of virions with co-precipitated material.

From the results of this study, the crude extraction protocol is a simple and effective method for the rapid extraction of infectious HAV from contaminated oysters, and can consistently yield reasonably high titres of infectious HAV compared to the more variable results obtained in the other methods used in this study. Further investigation would be useful to determine whether HAV extracted by this method will be sufficiently purified from PCR inhibitors found in oyster tissue to allow sensitive detection by RT-PCR.

Chapter 5: Accumulation of hepatitis A virus by oysters in a laboratory aquarium

5.1 INTRODUCTION

Bivalve molluscs obtain their food by filter feeding, a process where small organic particles are selectively filtered from the water and trapped in secreted mucous strands (DiGirolamo *et al.*, 1977; Eyles, 1981). Pathogenic bacteria and viruses can also be ingested by this method, and in environmental waters contaminated with human faecal effluent, bivalves may rapidly become contaminated and unfit for human consumption. As a result, consumption of bivalve molluscs has historically been associated with outbreaks of foodborne disease throughout the world, particularly due to enteric viruses such as HAV and norovirus (Grohmann *et al.*, 1981; Halliday *et al.*, 1991; Ang, 1998; Conaty *et al.*, 2000). Subsequently, many studies have been performed in laboratory aquaria to investigate the rate and mechanisms by which bivalves accumulate, retain and purge viruses (Di Girolamo *et al.*, 1975 and 1977; Bedford *et al.*, 1978; Eyles, 1981; Bosch *et al.*, 1995; Kingsley and Richards, 2003). Such studies have also revealed the ability of bivalves to accumulate viruses to a higher concentration than in the surrounding water.

The rate and efficiency with which oysters filter water and accumulate or purge contaminants varies depending on environmental factors such as the quality, temperature, turbidity and salinity of the water, as well as the species, age and size of the oyster (Eyles, 1981; Richards, 1988).

In the environment, viruses are often associated with sediment or other particulate matter, which protect against environmental extremes and assist in dissemination of the virions throughout estuaries and mollusc growing areas. Early research showed that association with marine sediment can increase the survival of viruses in seawater, and the presence of suspended solids in seawater can increase the incidence of virus bioaccumulation by bivalves (Metcalf *et al.*, 1979; Landry *et al.*, 1983). For example, Eyles (1981) observed an approximate 1000-fold greater uptake of cell-associated poliovirus by oysters than a purified poliovirus suspension.

The filter-feeding accumulation of viruses is a preferred method for contaminating oysters in the laboratory because it mimics the natural accumulation that occurs in the environment and under favourable conditions can achieve a high concentration of virus within the oyster tissue in a relatively short period. Laboratory aquaria used to investigate virus accumulation by bivalves may either be a recirculating system where contaminated water is pumped continuously around the aquarium throughout the experiment, or a flow-through system where sterilised seawater is continuously contaminated and pumped into the aquarium, then removed at the same rate to a holding tank for disinfection and discard. A flow-through system has advantages over a recirculating system due to the continual addition of fresh seawater, and is therefore closer to the environmental conditions of bivalves. Even so, recirculating aquaria have been shown to be effective in contaminating bivalves with viruses (Eyles, 1981; Kingsley and Richards, 2003). Flow-through systems may require large amounts of water during experiments, often pumped continuously direct from the sea. The

proximity of a laboratory to the water source may cause difficulty in the supply of seawater, while suitable sterilisation and disposal methods are required for the large volume of waste water generated.

The aim of this study was to contaminate oysters with HAV in a recirculating aquarium for the purpose of later treating the oysters with HPP. A requirement of the method was the ability to contaminate oysters with a high titre ($\geq 1 \times 10^4$ TCID₅₀/g oyster) of HAV, to ensure that any significant inactivation resulting from HPP would be measurable by the cell culture infectivity assay. Another requirement was that variation in accumulated HAV titre between oysters should be as low as possible to ensure accurate and repeatable results in the quantification of HAV inactivation following HPP.

5.2 METHOD

5.2.1 Aquarium, seawater and oysters

A 5L glass beaker was filled with 2 L seawater and a partly submersed aquarium pump for the virus accumulation study. A polypropylene mesh was placed on the bottom of the beaker so that oysters would sit approximately 1 cm off the base. Seawater was aerated in the beaker by the exhalent flow of the pump, which generated air bubbles at the water surface. The pH of seawater ranged between 8.50 and 8.70, and water temperature, measured numerous times each day throughout the trials, ranged between 15.5 and 22.5°C. Seawater was run through the system for at least 24 h prior to introducing oysters, and changed daily until contaminated with virus.

Commercially grown and harvested Pacific oysters were transported from the oyster farm, located in an estuary in southern Tasmania, Australia, to the Food Science Australia laboratory in Werribee (Melbourne), Victoria, Australia, on ice by overnight air freight. Oysters were not in direct contact with ice during shipping so that the effect of the low temperature during transportation was minimised. To ensure recovery from adverse handling conditions that may have occurred during transport, oysters were maintained in an uncontaminated recirculating system for between 2-5 days prior to each trial, as specified. Upon arrival in the laboratory, oysters were lightly scrubbed and rinsed under cold tap water prior to introduction to the system. Rubber bands were tied around two oysters to secure the valves shut prior to freezing at -70°C in sealable plastic bags for future analysis as uncontaminated controls.

5.2.2 First trial

This first trial was designed to test the ability of oysters to accumulate HAV to a detectable level during a 24 h period of exposure to HAV. Two oysters of similar size were placed in the beaker and left to acclimatise for two days. During this time oysters were fed a microalgae mixture consisting of 25% *Isochrysis*, 20% *Pavlova*, 20% *Tetraselmis*, 30% *Thalassiosira weissflogii*, and 5% *Nannochloropsis* (Instant Algae Shellfish diet 1800®; Reed Mariculture, U.S.A.), formulated for feeding bivalve molluscan shellfish (Reed, 2003). A total of 6 ml (approximately 1.2×10^{10} cells) microalgae were added to the beaker (containing two oysters) per day at regular intervals, and oyster feeding was monitored by inspecting for production of faeces and clearing of the water.

To contaminate oysters, 1 ml microalgae (approximately 2×10^9 cells) was mixed with 1.1×10^7 TCID₅₀ HAV, and added to the circulating seawater over a 7.5 h period, after which a sample of seawater was mixed with a 1:100 dilution of antibiotic/antimycotic solution and assayed for HAV titre. After a total exposure period of 24 h, oysters were removed from the water and the crude virus extraction method, described in Chapter 4, was applied to contaminated oysters as well as thawed uncontaminated oysters.

5.2.3 Second trial

A number of modifications were made to the method used in the first trial to increase the titre of HAV accumulated by oysters. A preliminary investigation was performed to ensure the health and feeding capability of oysters was optimal. Oysters were divided between 3 beakers containing seawater, with each oyster again being placed on polypropylene mesh to sit above the base of the beaker. Magnetic stirrers were used for water circulation in each beaker, and 0.5 ml (approximately 1×10^9 cells) microalgae added to each beaker to stimulate feeding. Oysters were observed regularly over a 2 h period, and 4 oysters that were observed pumping throughout this period were removed. Two oysters were each transferred to two identical beakers for a 5 day acclimatisation period, after which a spiked microalgae mixture was prepared with approximately 1.1×10^7 TCID₅₀ HAV stock and 1 ml microalgae (approximately 2×10^9 cells). After 1 h incubation on ice to promote attachment of virions to algae cells, the mixture was added to seawater in each beaker over a 4 h period, then left to circulate. An oyster was removed from each beaker after accumulation periods of 8 h and 24 h,

and the crude extraction method applied to contaminated oysters and thawed uncontaminated oysters.

Water samples were taken from each beaker at 0 h (immediately prior to contamination), 4 h (immediately after the final volume of virus was added to the water), 8 h (immediately after the first oyster was removed), and 24 h (immediately after the second oyster was removed), and treated with 1:100 antibiotic/antimycotic solution prior to quantitative analysis.

5.2.4 Crude virus extraction method

The crude extraction method described in Chapter 4 was used to extract HAV from infected oysters, with the following modifications for live oysters:

Oyster shells were scrubbed and disinfected (Virkon®; Antek, U.K.), then rinsed with sterile deionised water. Each oyster was shucked into a separate sterile blender bag and homogenised with a stab mixer for 30s. The homogenate was weighed and diluted 1:10 (w/v) in glycine buffer, then mixed well to promote dissociation of virions from oyster particulates. A 40 ml aliquot of the homogenate was centrifuged at 15,000×g for 1 h at 4°C to pellet debris, and the pH of the virus-containing supernatant was lowered to 7.0 (± 0.3) with 1 M HCl. A 5 ml aliquot was removed for analysis and mixed with 1:50 antibiotic/antimycotic solution. Contaminated oysters were analysed by tissue culture infectivity assay as well as quantitative real-time RT-PCR (qRT-PCR) for comparison.

5.2.5 RNA extraction

A 0.14 ml aliquot of extracted HAV was transferred to a microcentrifuge tube containing 3.5 µg RNase, and the sample incubated to degrade exogenous RNA that may affect the results of downstream qRT-PCR quantification. RNase was quenched upon addition of 560 µl lysis buffer AVL, supplied in the QIAmp® Viral RNA extraction kit (Qiagen, Australia). Prior tests confirmed that buffer AVL quenched the RNase activity without noticeable degradation to sample RNA. The remainder of the protocol was followed to manufacturer's instructions, and purified HAV RNA was eluted in 80 µl RNase-free water containing 0.04% sodium azide (Qiagen).

5.2.6 Quantitative RT-PCR

Quantitative RT-PCR was performed using the Bio-Rad iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad, U.S.A.). Reaction mix consisted of 12.5 µl 2× reaction mix, 3 µl of 0.3 µM forward and reverse primers (JotFor and JotRev, respectively), 0.5 µl reverse transcriptase, and 6 µl template RNA (total reaction volume of 25 µl).

Primers used were originally described by Jothikumar *et al.* (2005), and produced an 89-base pair (bp) amplicon. The primer sequences are as follows:

Forward: 5' GGTAGGCTACGGGTGAAAC 3';

Reverse: 5' AACAACTCACCAATATCCGC 3'.

Each reaction was transferred to duplicate wells on a 96-well PCR plate. Quantitative RT-PCR was performed on the Bio-Rad iQ5 iCycler, using the following cycle protocol: reverse transcription at 50°C for 10 min, reverse transcriptase inactivation for 5 min at 95°C, 40 cycles of 95°C, 10 s; 55°C, 20 s; and 72°C, 15 s; then melt curve cycling at 95°C for 1 min, 55°C for 1 min, and 80 cycles for 10 s each starting at 55°C and increasing by 0.5°C each cycle to 95°C.

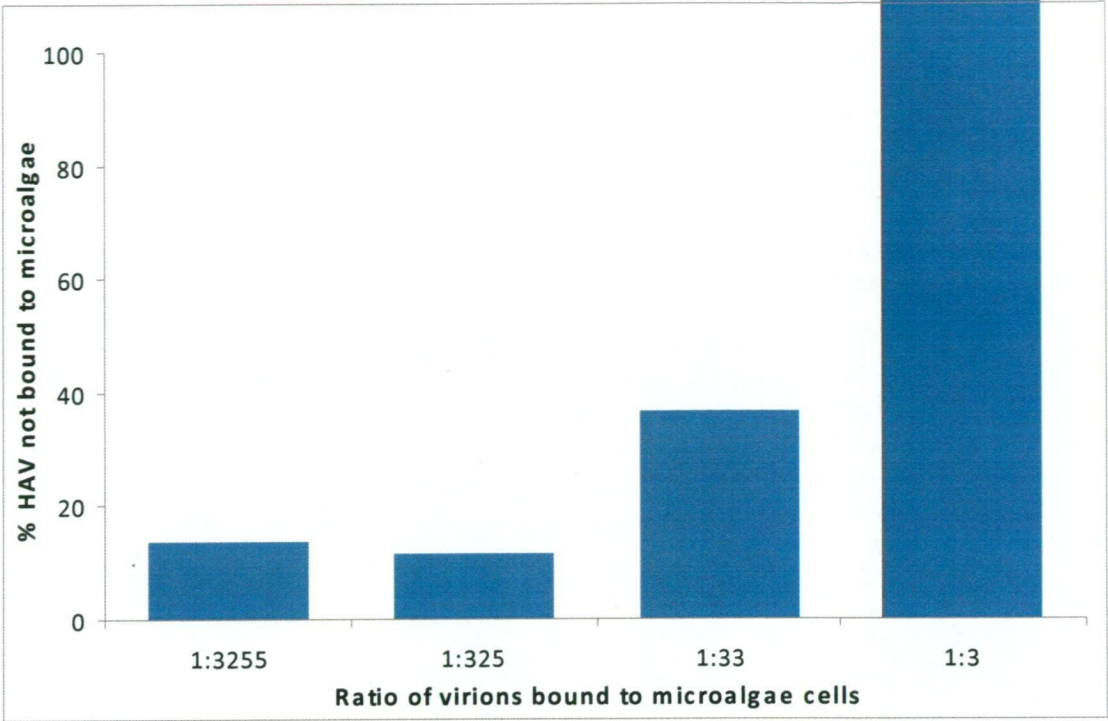
Data analysis was performed using the data analysis software provided with the Bio-Rad iQ5 iCycler. Fluorescence generated by the SYBR Green dye in each PCR reaction was measured at the completion of each 72°C primer extension cycle, and was used to calculate the reaction's cycle threshold (Ct) value, indicating the number of cycles taken for the fluorescence to reach an arbitrary threshold. The threshold chosen was determined by default by the iCycler software in each run, and in some cases was adjusted to produce a better fit to the standard curve. Ct is not necessarily an integer, and the exact value may be calculated by the iCycler software if the threshold falls between cycles.

5.2.7 Standard curve

A duplicate 10-fold HAV dilution series was prepared in sterile PBS from 1:1 to 1:100,000. Each duplicate dilution was quantified by triplicate infectivity assays, and the average of the six replicate assays was used as each dilution's titre. RNA was extracted from HAV dilutions and a qRT-PCR performed as already described to generate the standard curve of Ct vs. Log₁₀ starting quantity, which in this case is the infectious HAV titre. The titre of infectious HAV in a sample can be calculated by marking its Ct value on the standard curve.

5.3 RESULTS

The amount of hepatitis A virions bound to microalgae cells was examined over a number of virion: cell ratios as a way to ensure maximum uptake of virus by oysters. Ratios of approximately 1:3, 1:33, 1:325 and 1:3255 virions: microalgae cells were examined. Eluting virions off microalgae cells was an inefficient process (<2% recovery in each ratio), so instead the amount of virions remaining unattached to cells prior to elution was measured. The amount of binding between HAV virions and microalgae cells was dependent on the ratio, with approximately 1:325 resulting in the lowest amount of unbound virus (11.8% of initial inoculum), thereby giving oysters the greatest opportunity to take up HAV (Figure 5.1). A ratio of approximately 1:325 was subsequently used for the trials. The highest amount of unbound virus was obtained in the 1:3 ratio (232%), making it more difficult for oysters to filter out the relatively small virions from the water. The reason for obtaining more than 100% recovery of virus is likely to be due to the dispersal of virus clumps within the sample.



Ratio	% unbound
1:3255	13.7
1:325	11.8
1:33	36.6
1:3	232

Figure 5.1. Percentage of hepatitis A virus (HAV) remaining unbound to microalgae cells.

5.3.1 Quantitative RT-PCR

5.3.1.1 Standard curve

Duplicate 10-fold dilution series of HAV were prepared in sterile PBS to generate a standard curve for qRT-PCR. The infectious titre of each dilution (TCID₅₀/ml) was converted to the log₁₀ infectious titre within the qRT-PCR reaction. A linear relationship was observed between log₁₀ infectious titre and Ct value, with the coefficient of determination (R^2) >0.990 between the 1:1 and 1:100,000 dilutions (Figure 5.2). The quantification limit of the infectivity assay is 29.5 TCID₅₀/ml undiluted HAV extract. The HAV titre in the 1:100,000 dilution is under the quantification limit of the infectivity assay, and has been designated with a titre of 2.98 TCID₅₀/ml, i.e. 1-log₁₀ below the titre of the 1:10,000 dilution.

5.3.2 First trial

A total of 1.1×10^7 TCID₅₀ HAV was added to the seawater over a 7.5 h period. After this accumulation period, the amount of infectious HAV remaining in the recirculating seawater was 2.1×10^5 TCID₅₀/2L (100 TCID₅₀/ml), or 7.6×10^5 TCID₅₀/2L (380 TCID₅₀/ml) measured by qRT-PCR.

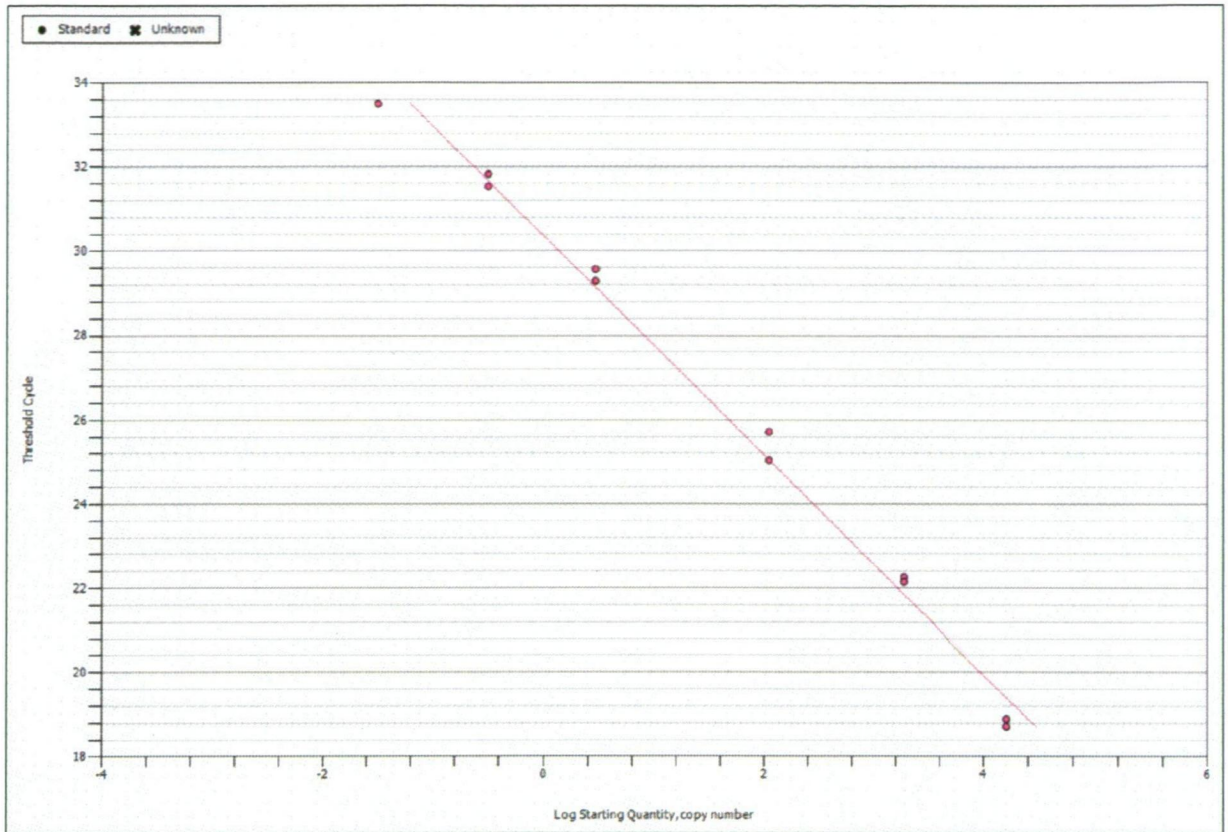


Figure 5.2. SYBR Green real-time quantitative RT-PCR standard curve for hepatitis A virus (HAV). R^2 : 0.992; Slope: -2.601; Y-intercept: 30.349.

After the 24 h accumulation period, HAV was detected in one of two oysters at 710 TCID₅₀/g by infectivity assay and 91.2 TCID₅₀/g by qRT-PCR. In the second oyster, contaminating HAV was below the quantification limit in the infectivity assay (limit 302 TCID₅₀/g oyster) and in qRT-PCR (limit 30.4 TCID₅₀/g oyster); yet the dissociation peak of HAV was detected by melt curve analysis during qRT-PCR (Figure 5.3).

The melt curve, or dissociation curve, is used in quantitative PCR applications when using SYBR Green as the fluorescing dye, and is a graph of the change in fluorescence ($-d(RFU)/dT$) with temperature (°C), generated during cycling that takes place immediately after the PCR reaction, where the temperature is incrementally increased between two set temperatures. A sudden drop of fluorescence is recorded when the amplicon's dissociation temperature is reached, and therefore the dissociation peak corresponds to the melting temperature (T_M) of the amplicon generated during from qRT-PCR. The T_M of the HAV amplicon generated by the JotFor/JotRev primer pair is $78 \pm 1.0^\circ\text{C}$, and the small peaks visible between 73 and 76.5°C in the non-inoculated negative control oyster sample and in samples recovered from contaminated oysters do not correspond with that of the HAV product. Rather, the small nature of the peaks and cluster of T_M values indicate the presence of non-specific product or primer-dimer, and were most likely to have been passed through the RNA extraction process from oyster tissue.

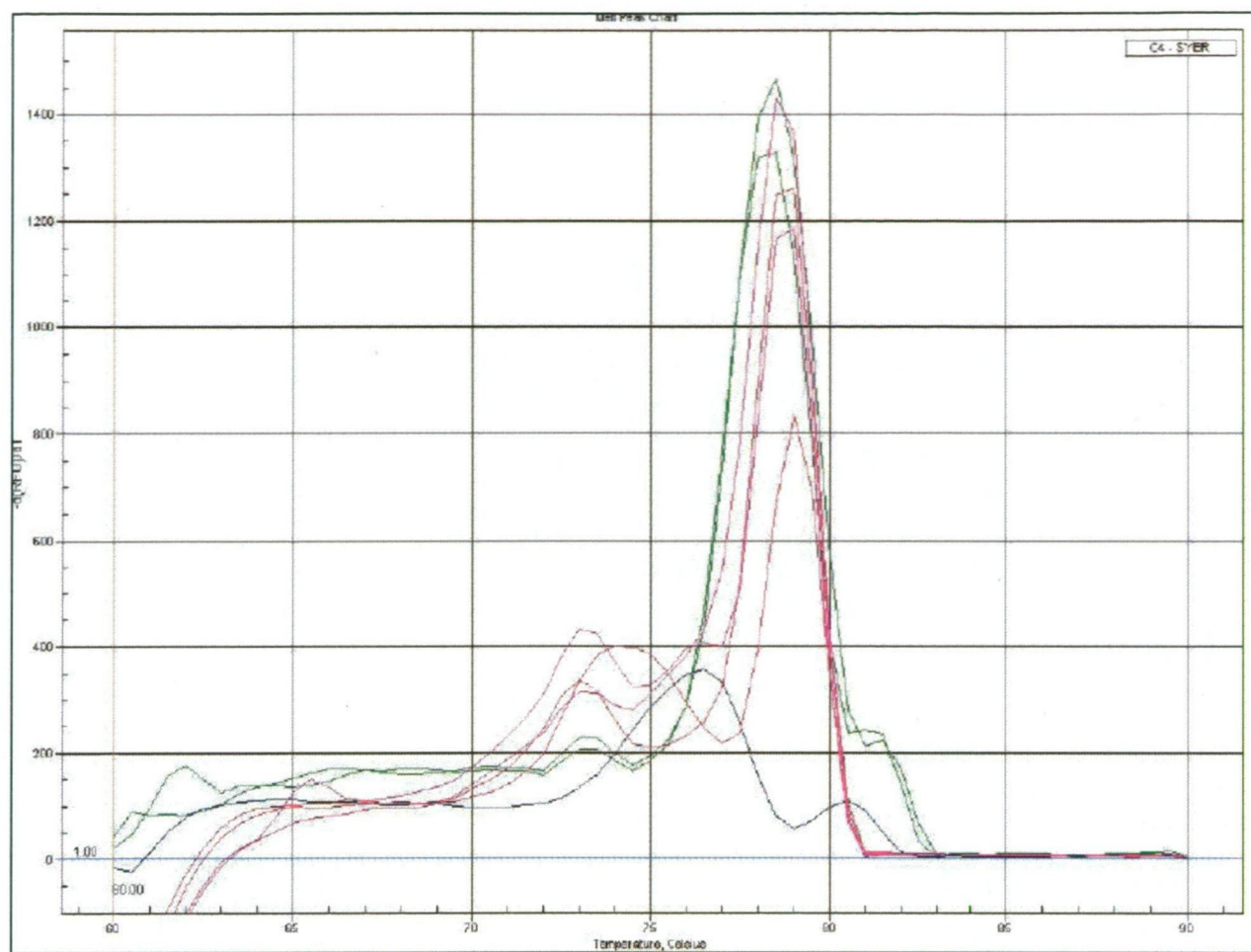


Figure 5.3. Melt curve of quantitative RT-PCR amplicons from trial 1 samples, showing hepatitis A virus (HAV) extracted from contaminated oysters (pink), buffered HAV stock (green), and oyster negative control, with HAV inoculum replaced with sterile PBS (blue). Expected HAV melting temperature is $78 \pm 1.0^{\circ}\text{C}$.

5.3.3 Second trial

A total of 1.1×10^7 TCID₅₀ HAV was added to each beaker over a 4 h period, and seawater was sampled from both beakers for HAV quantification immediately before the initial contamination, and then after 4, 8 and 24 h accumulation.

An oyster was removed from each beaker after 8 h and 24 h accumulation. In beaker 1, 5.0×10^2 TCID₅₀/g infectious HAV was detected in the oyster removed after 8 h accumulation, but infectious HAV was not detected in the oyster removed after 24 h accumulation. Neither oyster from beaker 2 contained a detectable amount of infectious HAV (Table 5.1).

Conversely, HAV was detected in all four oysters by qRT-PCR, and generally in higher numbers than within oysters in the first trial. The HAV titre within oysters in beaker 1 decreased from 1.7×10^2 TCID₅₀/g oyster by a small amount between 8 h and 24 h, but increased by approximately 1-log₁₀ from 5.0×10^1 TCID₅₀/g oyster during this same period within oysters in beaker 2.

HAV concentration within seawater sampled from either beaker peaked after 4 h of contamination, then fell to be undetectable in beaker 1 at 24 h, and in beaker 2, was at 34.1 TCID₅₀/ml, just above the quantification limit of the infectivity assay (Table 5.1).

Table 5.1. Titre of hepatitis A virus (HAV) in seawater (TCID₅₀/ml) and in oyster (TCID₅₀/g) in the second trial. Total HAV added to 2 L seawater was 1.1×10⁷ TCID₅₀. Limit of quantification in oysters: 302 TCID₅₀/g (infectivity assay), 30.4 TCID₅₀/g (qRT-PCR); infectivity assay limit of quantification in seawater: 30.2 TCID₅₀/ml. Mean of triplicate samples shown.

Time (h)	Infectivity assay				qRT-PCR	
	seawater		oyster		oyster	
	Beaker 1	Beaker 2	Beaker 1	Beaker 2	Beaker 1	Beaker 2
0	ND	ND	ND	ND	ND	ND
4	1.8x10 ²	8.8x10 ¹	-	-	-	-
8	7.1x10 ¹	7.1x10 ¹	5.0x10 ²	ND	1.7x10 ²	5.0x10 ¹
24	ND	3.4x10 ¹	ND	ND	1.4x10 ²	5.2x10 ²

‘ND’ denotes not detected; ‘-’ denotes not tested for

5.4 DISCUSSION

The aim of this study was to contaminate oysters with a high enough titre of HAV so that later inactivation studies with HPP would ensure that any significant inactivation could be quantified. The results of this study showed that HAV was accumulated by oysters, but not to the level required for HPP inactivation experiments.

Many factors contribute to the pumping ability of oysters, including the general health of the oyster, and the salinity, temperature and quality of the seawater (Eyles, 1981; Bird, 1994). In this investigation, oysters were harvested from their estuarine water, sorted by size and flown directly from the harvester on the same day, arriving at the laboratory the following morning. Oysters were shipped with an ice pack to lower the temperature during transportation, but were not in direct contact with ice. To ensure recovery from adverse handling conditions that may have occurred during transport, oysters were maintained in an uncontaminated recirculating system for between 2-5 days prior to the trial. During this time, regular observations were made throughout each day to confirm oysters were alive and filter feeding efficiently, such as checking for open valves, production of faeces and pseudofaeces, and water clearing. For the majority of each trial, the valves of oysters were observed to be open; faeces and pseudofaeces were cleaned from the base of the beakers daily during the acclimatisation period; and the water visibly cleared of microalgae throughout the day and overnight, indicating oysters were healthy and pumping effectively. This indicated the low uptake of virus was probably not due to the ill-health of the oysters.

Water temperature that is too high or low or changes too rapidly can affect the pumping ability of oysters, or can even induce spawning (Bird, 1994). The oysters used in this study were harvested from the estuarine waters of Southern Tasmania. Water temperature data for this harvest area indicate a variation between a low of 6.5 and a high of 24.1°C between February 2003 and June 2006, with an average of 18.1°C during the warmer months (October to March) and 11.0°C during the colder months (April to September) (R. Brown, personal communication). The temperature of the seawater within aquaria in this study was monitored regularly each day, and varied between 15.5 and 22.5°C throughout the trial. Water temperature variations of no more than 1°C per hour are recommended in oyster depuration systems by Bird (1994) and were not exceeded during this study. Oysters were observed pumping at all temperatures throughout this range, and were not observed to be stressed by temperature variations they were exposed to in the laboratory.

In a study of persistence of HAV in Eastern oysters (*Crassostrea virginica*) Kingsley and Richards (2003) placed single oysters in beakers containing 9×10^4 PFU HAV, and allowed accumulation to occur over a 16 h period. Viruses were then extracted from shellfish tissue using a viable virus extraction method almost identical to the crude extraction method described in Chapter 4, and used in this study. An average of 3.4×10^4 PFU was detected within the oysters immediately after the accumulation period, using a plaque assay to quantitate infectious virus. The authors explained that the extraction method does not purify plaque assay inhibitors and cytotoxic substances from the HAV extract, which is possibly why only 37% of the initial HAV stock was detected in the oyster immediately after

accumulation. Whilst this undoubtedly played a part in the discrepancy, the results obtained in this current study suggest that oysters may not accumulate the entire virus culture within a closed recirculating system. Of course, oyster species and environmental conditions play a part in virus uptake, but in the present study <1% of the initial infectious HAV titre was detected in oysters.

Calci *et al.* (2005) used a flow through contamination system to contaminate 22 oysters with HAV, which were then pooled together for challenge testing with HPP. Using the same viable virus extraction method described by Kingsley and Richards (2003) and a plaque assay to quantitate levels of infectious virus, the researchers reported an accumulation rate of $>10^5$ PFU per oyster, with accumulated titres remaining relatively consistent between oysters in the three replicate trials performed. Use of a flow through system may have assisted oysters in the accumulation of a consistently high titre of HAV. The flow-through contamination system mimics the environmental conditions of oysters better than a recirculating system by providing a constant supply of fresh water, food and virus to the oysters. A recirculating system was chosen for use in the present study because of the ease of aquarium setup and disinfection of a smaller water volume, and because of the adequate accumulation achieved in previous studies that used recirculating systems for contamination of bivalve molluscs (Eyles, 1981; Kingsley and Richards, 2003).

Eyles (1981) suggested that in a recirculating system the virions available to be taken up by an oyster most efficiently will be accumulated first. An example of this is a virion adsorbed to an organic particle ingested for food by an oyster. As

a result, those virions considered less 'attractive' to the oyster, for example unbound virions, may remain in the water. Virus accumulation would therefore be expected to peak initially and then slow over time, as seen in the second trial of the current study where the concentration of infectious HAV in oysters declined after the initial spike in accumulation at 8 h. In a flow-through system this would not be expected, as the virus population in the water is being constantly supplied and renewed.

The large amount of virus left unaccounted for during the trials was of concern. For example, in the second trial, although 1.1×10^7 TCID₅₀ HAV was added to each beaker, < 2% and < 1% of this was detected in the seawater and in the oyster, respectively, sampled after 8 h accumulation. Furthermore, no infectious HAV was detected in either the oyster or the seawater after 24 h accumulation.

Bedford *et al.* (1978) investigated the accumulation of radioactive-labelled reovirus by the rock oyster (*Crassostrea glomerata*) from seawater in a beaker. To examine non-accumulated virus losses, an oyster was killed by immersion in liquid nitrogen, thawed, and placed in seawater contaminated with reovirus. A substantial quantity of virus was removed from the water by adsorption to the oyster shell. In a beaker containing only contaminated seawater, approximately 50% of the added reovirus was removed from the seawater and adsorbed to the beaker walls after approximately 24 h.

This migration of virions from the water to solid surfaces within the beaker may provide an explanation for the inability to detect HAV after 24 h accumulation. In the present study, considerable clearing of microalgae from the seawater was observed after the 24 h accumulation period, and was initially assumed to be the result of accumulation by oysters. On closer inspection, however, the surfaces of the oyster shells, aquarium pump and beaker walls were discoloured and slimy due to adsorption of microalgae. Virions associated with microalgae were presumably also becoming adsorbed to the surfaces within the aquarium, meaning the seawater contained a gradually decreasing titre of virus available for accumulation over the course of the 24 h period. With a decreasing HAV concentration in the seawater, a purging effect by oysters may be expected, resulting in the decrease of HAV concentration within oysters over the 24 h period.

The study by Bedford *et al.* also revealed that adsorption to beaker walls could be reversed by the addition of bovine serum albumin (BSA) to the seawater at a concentration 25 times that of the virus. BSA was not added to seawater in the current study due to the unknown effects it may have on shellfish filter feeding, and the limited time available to test these effects in the laboratory.

Quantitative RT-PCR detected HAV in all oysters placed in contaminated seawater, even when undetectable by infectivity assay. The quantification limit was 302 TCID₅₀/g oyster for the infectivity assay and 30.4 TCID₅₀/g oyster for qRT-PCR. This result may be interpreted as qRT-PCR having superior sensitivity over the traditional infectivity assay; or that qRT-PCR does not correlate with levels of infectious HAV in oysters. Virions may contain intact RNA, yet be non-infectious due to damage to the outer capsid preventing infection. In such a case, the virus would not be detected by traditional infectivity assay, but could be detected by RT-PCR. This limitation of qRT-PCR has been addressed previously (Chaves *et al.*, 1994; Reynolds *et al.*, 1997; Richards, 1999; Hewitt and Greening, 2006), and must be considered during viral inactivation studies and in environmental sampling. This could well be the reason for the disparity in the current study between infective titres and qRT-PCR titres in oysters, but the difference in sensitivity of the two quantitative techniques must also be taken into account. Quantitative RT-PCR is a more sensitive assay than the infectivity assay used in this study, and can potentially detect lower levels of HAV in oyster samples than the current cell culture technique.

Further improvement to the assay design is recommended, as the concentration of detected HAV was close to or below the quantification limit of the qRT-PCR assay in the oyster samples. Further sample purification and/or concentration should be considered to increase the assay's quantitative sensitivity; in particular, a purification step to selectively purify infectious virions would be of most benefit.

The aim of this research was to contaminate live oysters with a high titre of infectious HAV by utilising the natural filter feeding ability of the oysters. It was anticipated oysters would concentrate HAV to a much higher concentration within oyster tissue than the surrounding water. However, the actual titres achieved in the oysters containing quantifiable infectious HAV were not high enough for a significant reduction in titre to be achieved upon subsequent HPP treatment.

One disadvantage in allowing oysters to naturally accumulate virus is that the accumulated titre will be unknown until after the experiments and lengthy infectivity assay is completed. One way to contaminate shellfish meat with a known quantity of virus is to inject it directly into the shellfish meat. This approach was used in a recent study to spike mussels within the shell for subsequent heat treatment and was effective in achieving a known high titre within mussel tissue (Hewitt and Greening, 2006). This method of injecting a known virus titre into shucked oyster meat was attempted prior to conducting this study, and was deemed inappropriate because within minutes the culture liquid was observed draining from the oyster again (unpublished results). A better option may be to shuck the oysters, homogenise the meat and then spike it with the required titre of virus.

Chapter 6: Development of a high pressure processing inactivation model for hepatitis A virus

6.1 INTRODUCTION

HPP is proving to be a popular processing tool for retail seafood products, and particularly shellfish, for several reasons. Firstly, *Vibrio* species, associated with a number of foodborne outbreaks following consumption of contaminated shellfish in the U.S.A., are sensitive to HPP (Styles *et al.*, 1991; Cook, 2003; Koo *et al.*, 2006). Secondly, the limited refrigerated shelf-life of oysters can be extended by high pressure treatment with little effect on sensory quality (He *et al.*, 2002) and the flavour of oysters may even be enhanced by pressure infusion of the salty liquor within the oyster shell into the meat (Hoover *et al.*, 1989). Thirdly, oysters can be shucked with appropriate high pressure treatment. Treatment at 241 MPa for 2 min shucked 88% of oysters (*Crassostrea gigas*), and pressurisation to 310 MPa with immediate depressurization upon reaching 310 MPa resulted in 100% shucking (He *et al.*, 2002). Shucking is a result of the pressure-induced denaturation of adductor muscles which hold the oyster shell tightly closed, and allows convenient removal of oyster meat without the need for shucking knives (He *et al.*, 2002; Cruz-Romero *et al.*, 2004).

HAV continues to cause outbreaks of serious illness attributed to consumption of contaminated foods, and particularly shellfish (Halliday *et al.*, 1991; Conaty *et al.*, 2000; Bosch *et al.*, 2001). This is largely due to bivalve molluscs accumulating microorganisms from the water during filter feeding.

The commercial availability of pressure-treated shellfish products in U.S.A. raises the question of the treatment's effectiveness on enteric viruses known to contaminate these foods. A number of studies have been performed to investigate the effects of HPP on enteric viruses and their surrogates. Kingsley *et al.* (2002) reported that HAV suspended in tissue culture medium (DMEM with 10% FBS) was reduced by approximately 7-log₁₀ PFU/ml following 5 min treatment with 450 MPa at ambient temperature. A surrogate for the unculturable human norovirus, feline calicivirus (FCV) was reduced from 7-log₁₀ TCID₅₀/ml to an undetectable level (10^{0.5} TCID₅₀) after 5 min treatment with 275 MPa or higher. In contrast, no reduction in infectious poliovirus (PV) titre was recorded after 5 min treatment with 600 MPa.

Calci *et al.* (2005) studied the effects of HPP on HAV in oysters that were artificially contaminated using a specially designed laboratory flow-through contamination system. The shucked oysters were subjected to 1 min treatments at varying high pressures at 9°C. HAV titre was reduced by <0.5-log₁₀ with 300 MPa, >1-log₁₀ with 350 MPa, and by >3-log₁₀ with 400 MPa.

These studies demonstrate the sensitivity of HAV to HPP; however salinity, temperature, treatment time and substrate can each affect the magnitude of microbial inactivation by HPP. As noted by Chen *et al.* (2005), pressure inactivation kinetics must be clearly understood to establish safe processing conditions and, as such, more studies assessing the effects of these factors on the inactivation of HAV are required. The need to keep the fresh appearance of pressure-treated oysters and the commercial requirement for high-throughput processing of product means that commercial treatment times longer than 5 min are probably not economically viable, so the need exists for kinetic inactivation data over a commercially relevant range of treatment times, i.e. 1-5 min.

Predictive microbial modelling involves the development of mathematical equations that describe the growth, survival or death of microorganisms in response to specific and controlled conditions. “Primary models” are used to describe the changes in microbial populations over time under constant conditions whereas “secondary models” describe the influence of environmental conditions (e.g. pressure, osmotic potential) on the parameters of the primary model (R.C. Whiting and R.L. Buchanan, Authors’ Reply to Letter, *Food Microbiol.* 10:175-177, 1993). HPP inactivation models have been developed for various bacteria and bacteriophages in different substrates (Chen and Hoover, 2003 and 2004; Bull *et al.*, 2005; Avsaroglu *et al.*, 2006); however a HPP inactivation model for HAV has not been published to date.

The aim of this research was to collect data describing the response of HAV to high pressure over a range of processing times, and at two salt concentrations that are representative of the water salinity in shellfish farming areas in Australia. Development of a HPP predictive inactivation model is then proposed, so that inactivation can be predicted by interpolation, without the need for further laboratory challenge testing. The model may benefit oyster processors wishing to make an informed decision regarding the implementation of HPP into their process line to increase the microbiological safety of their product.

6.2 METHODS

6.2.1 Sample preparation – Buffered samples

Stocks of HAV were prepared using the freeze-thaw method described in Chapter 3. HAV was suspended in DMEM (15 parts per thousand (ppt) salt) and in DMEM containing dissolved Red Sea marine salt mixture to increase salt concentration to 30 ppt. These concentrations were chosen to be representative of Australian coastal seawater conditions. For example, Australian coastal seawater and estuarine environments often range between 30 and 34 ppt, but estuaries especially may drop to as low as 15 ppt during periods of high rainfall. Salinity of the solutions was calculated by measuring the density and temperature of the medium, and then using these two values to calculate the salt concentration from a conversion chart (Bird, 1994).

Approximately 1.6 ml HAV stock was used to fill screw-cap cryotubes (Nalgene) leaving minimal air bubbles within the tube. The final titre of HAV used in HPP treatments was approximately 5-log_{10} TCID₅₀/ml.

6.2.2 Sample preparation – Spiked oyster homogenate

Commercially grown and harvested Pacific oysters (*Crassostrea gigas*) were transported from the oyster farm, located in an estuary in southern Tasmania, Australia, to the Food Science Australia laboratory in Werribee (Melbourne), Victoria, Australia, on ice by overnight air freight. Upon arrival in the laboratory, oysters were lightly scrubbed and rinsed under cold tap water.

The intracellular ionic strength of oysters was adjusted to the salt concentrations of DMEM during data collection (i.e. 15 ppt and 30 ppt) by placing live oysters randomly in one of two beakers initially containing 30 ppt seawater. Oysters were kept approximately 1 cm off the base of each beaker by polypropylene mesh, while a magnetic stirrer controlled water circulation. Water was replaced with 30 ppt seawater daily in one beaker, and in the second beaker water was replaced twice daily with fresh seawater of decreasing salinity in 5 ppt increments. Oysters in this beaker were exposed to 15 ppt seawater for at least two days prior to homogenisation. One millilitre (approximately 2×10^9 cells) Instant Algae Shellfish diet 1800® was fed to oysters twice daily (total 2ml per day), and feeding was monitored by inspecting for production of faeces and clearing of the water.

After the adjustment period, oysters were removed from the aquaria and shucked into a blender bag for each salt concentration. Excess liquid was poured off, then oysters from each salt concentration were homogenised with a stab mixer (model SM 8; Tiffany, Melbourne, Australia) until smooth homogenates were formed.

One hundred grams of homogenate was spiked with HAV stock to achieve a titre of approximately 3×10^5 TCID₅₀/g. After thorough mixing, homogenate was incubated on ice for 30 min to promote adsorption of virions to oyster solids. Ten gram sub-samples of spiked homogenate were heat-sealed within blender bag pouches containing minimal air bubbles.

6.2.3 High pressure treatment

Samples were loaded into a plastic screw-capped container, and packed on ice within an insulated rigid box for overnight air transport to the Food Science Australia laboratory in North Ryde (Sydney), New South Wales, Australia for HPP and kept at 4°C. Untreated HAV controls were transported to the North Ryde laboratory as described above and stored with treated samples, or remained in the Werribee laboratory at -70°C and 4°C to quantitate any potential losses in titre resulting from storage or transport. An uninoculated oyster homogenate control also transported to the North Ryde laboratory was not pressure-treated.

Pressure treatments were performed in a 2 L HPP unit (Flow International Corporation, Kent, Washington, U.S.A.) at the Food Science Australia laboratory in North Ryde using water as the pressurisation fluid. The initial temperature of all treatments was ambient temperature (approximately 18-20°C). Pressure come-

up time was less than 10 s, and depressurisation occurred in less than 5 s. Buffered samples were treated with 300, 400 and 500 MPa for between 60s and 600s, and spiked homogenate samples were treated with 325, 375, 425, 450 and 475 MPa for between 60 and 510 s. Pressure treatments were performed on single samples at a time, and replicated on later days.

The temperature within the high pressure vessel increases uniformly with the increasing pressure, and is referred to as adiabatic heating. Although the temperature of the vessel within this 2 L unit could not be measured, it is estimated to be approximately 2-3°C per increase of 100 MPa. To assure the expected increase in temperature would have no effect on HAV infectivity, HAV stock was incubated at 40°C for 30 min. No loss of titre was observed (results not shown).

6.2.4 HAV quantitative analysis

An enzyme immunoassay, modified from Borovec and Uren (1997), was used to determine infectious HAV titre. For quantification of buffered samples, a 1:2 (v/v) dilution of sample was analysed in the assay, increasing the assay's detection limit to 29.5 TCID₅₀/ml. For spiked homogenate samples, a 1:4 (v/v) dilution was the lowest that would allow a readable assay (detection limit 59 TCID₅₀/ml). Any lower dilutions caused damage to tissue culture cells and prevented colour generation in infected wells for quantification.

6.2.5 Model development

Data analysis and model development from the buffered sample data was performed in Microsoft® Excel to determine relationships between variables and to subsequently fit the data to the model form developed. The independent variable was the survival ratio (LogS), the term given for the logarithm of the remaining infectious fraction of HAV at a particular treatment time. Using the Solver routine in Microsoft® Excel, models were fitted to data by minimisation of the root mean square of the differences between the fitted value and corresponding observed value of LogS.

The Weibull model (Peleg and Cole, 1998), of the form:

$$\text{LogS} = -bt^n \quad (1)$$

was initially selected as a primary model to fit inactivation data following the approach of Mattick *et al.* (2001). The value for b determines the scale of the curve and the value for n determines the curve's shape (Peleg and Cole, 1998). Values for b and n were determined by using the Solver routine in Microsoft® Excel to minimise the residual sum of squares (square root of the difference between LogS (experimentally observed) and LogS (predicted by the model)) for all data in each combination of pressure and salt concentration, by iteratively changing values for b and n . If errors occurred in the convergence of Solver, a very small positive value (e.g. 1.0×10^{-10}) was used for 0 time.

When attempting to describe the parameters b and n as a function of pressure and salt concentration (i.e. to develop a secondary model), it was found that no simple

relationships existed between the parameters (b , n) and the independent variables (pressure, salt). In particular, it was noted that fitted values of n (describing the curvature in the inactivation rate data) could indicate that the inactivation curve was concave upwards ($n < 1$), concave downwards ($n > 1$), or that no inactivation was recorded ($n = 1$), without consistent relationship to pressure or salt concentration. To simplify the modelling, it was hypothesised that all inactivation was essentially log-linear, i.e. that curvature in the $\log S$ vs. time response data was due to uncontrolled factors in the experiments. The inactivation curve data were refitted to generate inactivation rates ($\partial \log S / \partial \text{time}$).

As a result, two approaches to modelling were evaluated. The first, describing the inactivation of HAV as log-linear and a function of pressure, treatment time and salt concentration, is a secondary model. Data from all combinations of pressure and salt concentration can be described in this model, and its development is described below. The second approach consists of six equations, each a Weibull model in the form described in equation (1), fitted to the data for a single pressure and salt concentration combination. Compounding the difficulty of model generation was that the inactivation rate response to pressure at 15 ppt salt appeared to be qualitatively different to the response at 30 ppt salt.

6.2.6 Development of a Secondary Model

Wherever possible, the simplest relationship describing the data was sought. Subsets of the data were plotted, relationships noted, and functions chosen to model the relationships. Relationships were then combined to develop a model structure that could describe the entire data set. That model structure was then re-fitted to

the entire dataset, using the Solver routine as described above to minimise the sum of the squares of the differences between the observed and the fitted values.

For treatment at 15 ppt, inactivation was found to be well described by the log-linear function:

$$\text{LogS} = \text{time} * \text{inactivation rate} \quad (2)$$

$$\text{where inactivation rate} = 1/(1.72551229 * \text{Pressure} - 917.680681)$$

The 30 ppt data were included in the model by addition of a term that models the relative rate of inactivation at 30 ppt compared to that at 15 ppt. That relative rate is a function of pressure, and was found to be described by:

$$\text{Relative rate (15 ppt/30 ppt)} = 0.0026 * \text{Pressure} - 0.781 \quad (3)$$

At pressures less than approximately 300 MPa, however, the above function predicts a relative rate less than zero. This would mean the virus titre was actually increasing during pressure treatment at one salt concentration, but decreasing during the same treatment at the other salt concentration. Assuming this is not possible, the minimum relative rate was therefore forced to zero. Equation (4) is an empirical function developed so that the relative rate in equation (3) would be forced to an asymptote of zero for pressures less than 300MPa:

$$\text{Correction} = 10^{-(300/(\text{Pressure}+0.001))^{10}} \quad (4)$$

For pressures of 300 MPa and above, equation (4) takes values very close to 1 but for values less than 300 takes values increasingly close to zero. Thus, the overall additional terms in the equation required for salt concentration of 30 ppt is:

$$(0.0026*Pressure-0.781)*(10^{-(300/(Pressure+0.001))^{10}}) \quad (5)$$

So that the model will include the correction factor when the salt concentration is 30 ppt, but not for 15 ppt, equation (5) is raised to the power of 0 when the salt concentration is 15 ppt but is raised to the power of 1 when the salt concentration is 30 ppt. This is achieved with the following empirical function:

$$(Salt\ concentration-15)/15 \quad (6)$$

which has the value of 0 when salt concentration is 15 ppt, and a value of 1 when salt concentration is 30 ppt.

Thus, the overall model for describing LogS as a function of salt concentration, pressure and treatment time is:

$$\text{LogS} = \text{Time}/[(a*Pressure - b)]/$$

$$[(c*Pressure-d)*(10^{-(300/(Pressure+0.001))^{10}})]^{((salt\ conc.-15)/15)}$$

The above equation was then refitted to the entire dataset for buffered samples, allowing the parameters a , b , c and d to be determined from the data using the Solver routine, as described above, which results in the following fitted model:

$$\text{LogS} = \text{Time} / [(1.7255 * \text{Pressure} - 917.68) / [(0.0026 * \text{Pressure} - 0.781) * (10^{-(300/(\text{Pressure} + 0.001))^{10}})]^{([NaCl] - 15)/15}] \quad (7)$$

Equation (7) describes the data for buffered samples at both 15 and 30 ppt salt concentrations. Alternatively, equation (2) can be used to describe 15 ppt salt concentration data only.

Inactivation data were also fit using the Weibull model, as described above in section 6.2.5.

6.2.7 Tests for model fit

Comparison of the models was performed using the sum of squared residuals (SS), coefficient of determination (R^2), bias factor (B_f), and accuracy factor (A_f). Squared residuals (square root of the difference between LogS (observed) and LogS (predicted)) were calculated for each data point, then added up to give the SS. The lower the value for SS, the smaller the difference is between the observed data and the data predicted by the model.

The R^2 indicates the amount of variability in the data able to be described by the model, and an R^2 close to 1.0 indicates that most of the variation in the data is described by the model.

The B_f and A_f were described by Ross (1996) to evaluate the performance of predictive models. Each measures the average difference between observed and predicted LogS values, with the difference being that B_f takes into account the sign of the difference (so that underpredictions by the model will cancel out overpredictions) to indicate whether the model systematically over or underpredicts the observed values, whereas A_f is calculated from absolute values and provides a measure of variability about the predicted response.

B_f is calculated by the following equation:

$$B_f = 10^{(\sum \log(\text{predicted}/\text{observed})/n)}$$

where n is the number of observations used in the calculation.

A_f is calculated by the following equation:

$$A_f = 10^{(\sum |\log(\text{predicted}/\text{observed})|/n)}$$

Values close to 1.0 are desired for both factors. A B_f larger than 1 indicates a fail-dangerous model, with predictions of inactivation larger than those actually observed, and if less than 1 the model is considered fail-safe; predicting lower inactivation than that observed in the treatment. The A_f is always equal to or larger than 1.0, indicating the factor, on average, with which predictions vary from the corresponding observations.

6.3 RESULTS

6.3.1 Buffered samples

Survival curves (LogS vs. treatment time) for pressure-treated HAV suspended in buffered media are shown in Figure 6.1.

HAV suspended in buffered media containing 15 ppt salt was reduced by approximately 1- \log_{10} and 2- \log_{10} TCID₅₀/ml after 600 s treatment with 300 and 400 MPa, respectively (Figure 6.1a). HAV was more sensitive to HPP at 15 ppt than at 30 ppt. Treatment with 300 and 400 MPa had little effect on the titre of HAV in media containing 30 ppt salt, with <0.50- \log_{10} TCID₅₀/ml reduction in titre after 600 s treatment (Figure 6.1b). A more pronounced reduction in titre was observed during treatment with 500 MPa at both salt concentrations; HAV was undetectable (<1.47- \log_{10} TCID₅₀/ml) after 300 s treatment in 15 ppt and after 360 s treatment in 30 ppt.

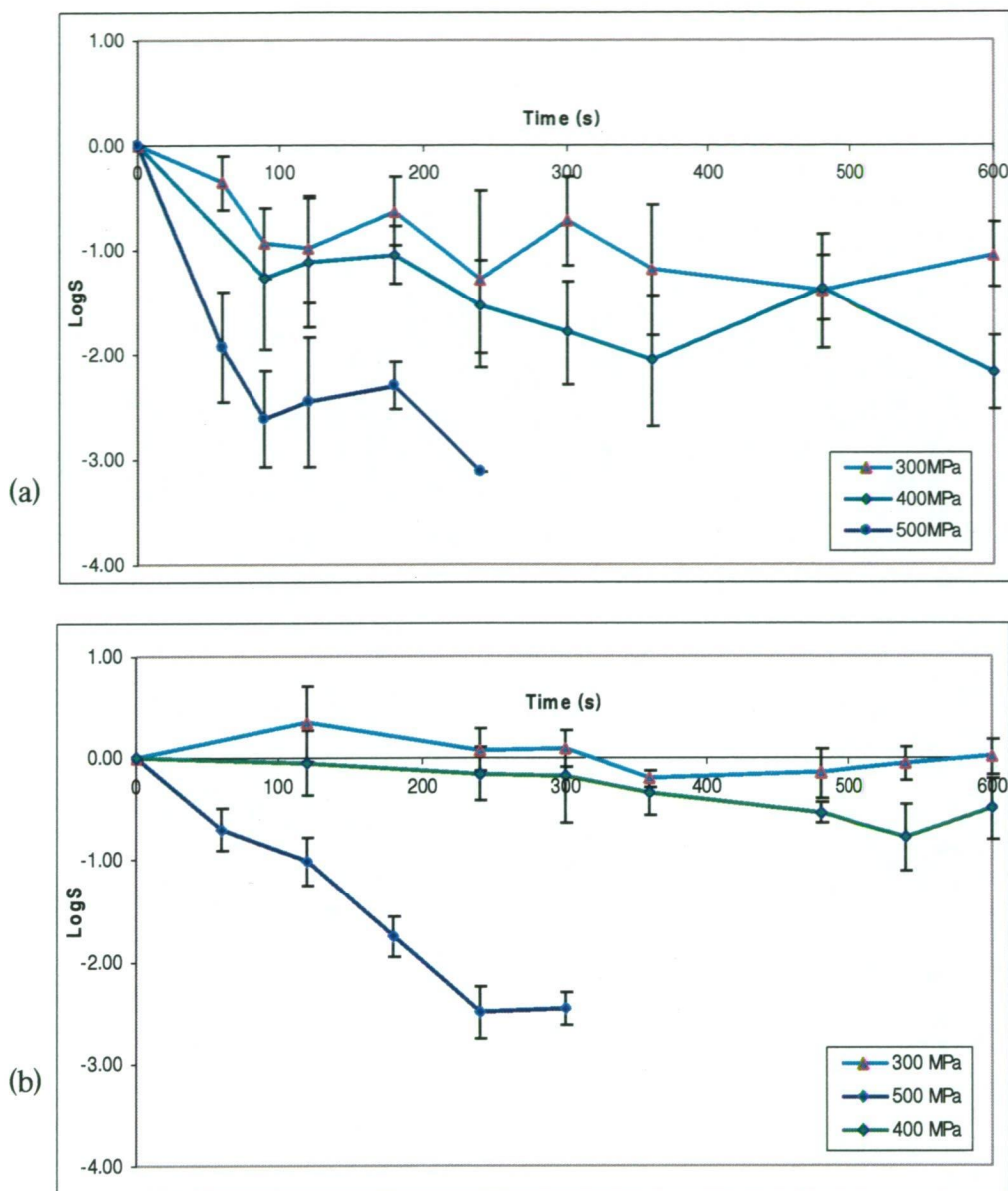


Figure 6.1. Survival curves of hepatitis A virus after pressure treatment at room temperature in buffered media containing (a) 15 ppt and (b) 30 ppt salt. Each data point is the average LogS value of triplicate quantitative assays for duplicate or triplicate samples. No infectious hepatitis A virus was detected ($<1.47\text{-log}_{10}$ TCID₅₀/ml) after 500 MPa treatment for 300 s in 15 ppt salt or 360 s in 30 ppt salt.

6.3.2 Fit and comparison of predictive models

The individual LogS values observed following HPP treatment were compared with those predicted by the individual Weibull models and the global log-linear model (Eqn. 7) for each combination of pressure and salt concentration (Figure 6.2 and 6.3).

The Weibull model takes on a tailing curve to describe the HPP data in 15 ppt salt, while the log-linear model predicts a straight line through the same data (Figure 6.2). In media containing 30 ppt salt, the Weibull model predicts inactivation with a straight line, tailing and a shoulder with the three levels of pressure (Figure 6.3), indicating the unpredictable nature of HAV inactivation in response to the treatments. The curve shapes are determined by the values for b and n in the Weibull function. Increasing b relates to a steeper curve and faster death, while the n value determines whether the curve has a tail, shoulder or is a straight line. At both salt concentrations, values for b increase with increasing pressure, but the value for n peaks at 400 MPa at both salt concentrations (Table 6.1). Furthermore, the magnitude with which values for b and n change in relationship to pressure level is erratic, and this relationship is best understood by plotting the values for b and n against salt concentration and pressure (Figure 6.4).

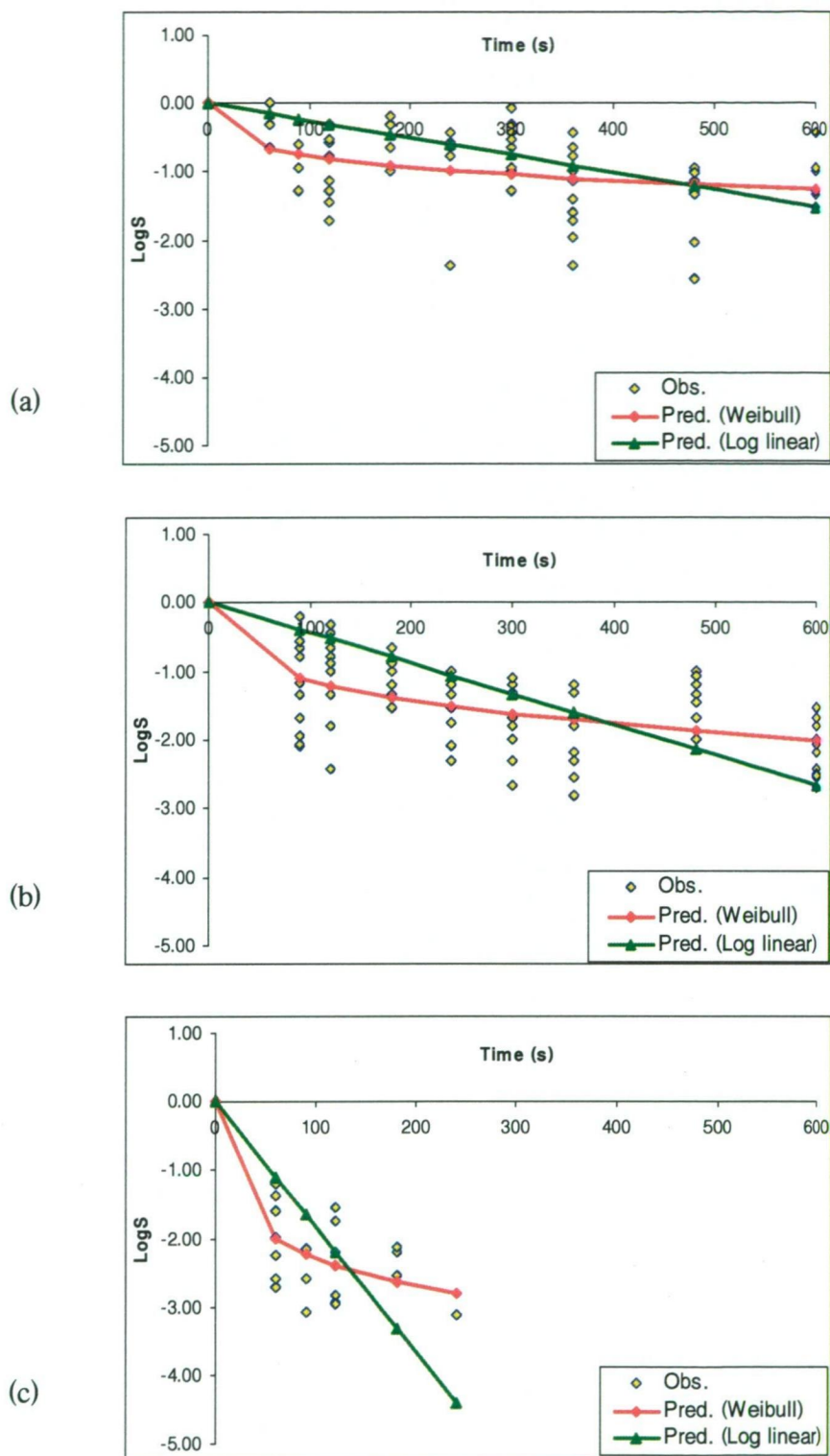


Figure 6.2. LogS values of hepatitis A virus predicted by the individual Weibull models (Weibull) and Equation 7 (Log linear), in comparison with the experimentally-derived LogS values (Obs.) for treatments with (a) 300 MPa, (b) 400 MPa and (c) 500 MPa in buffered media containing 15 ppt salt.

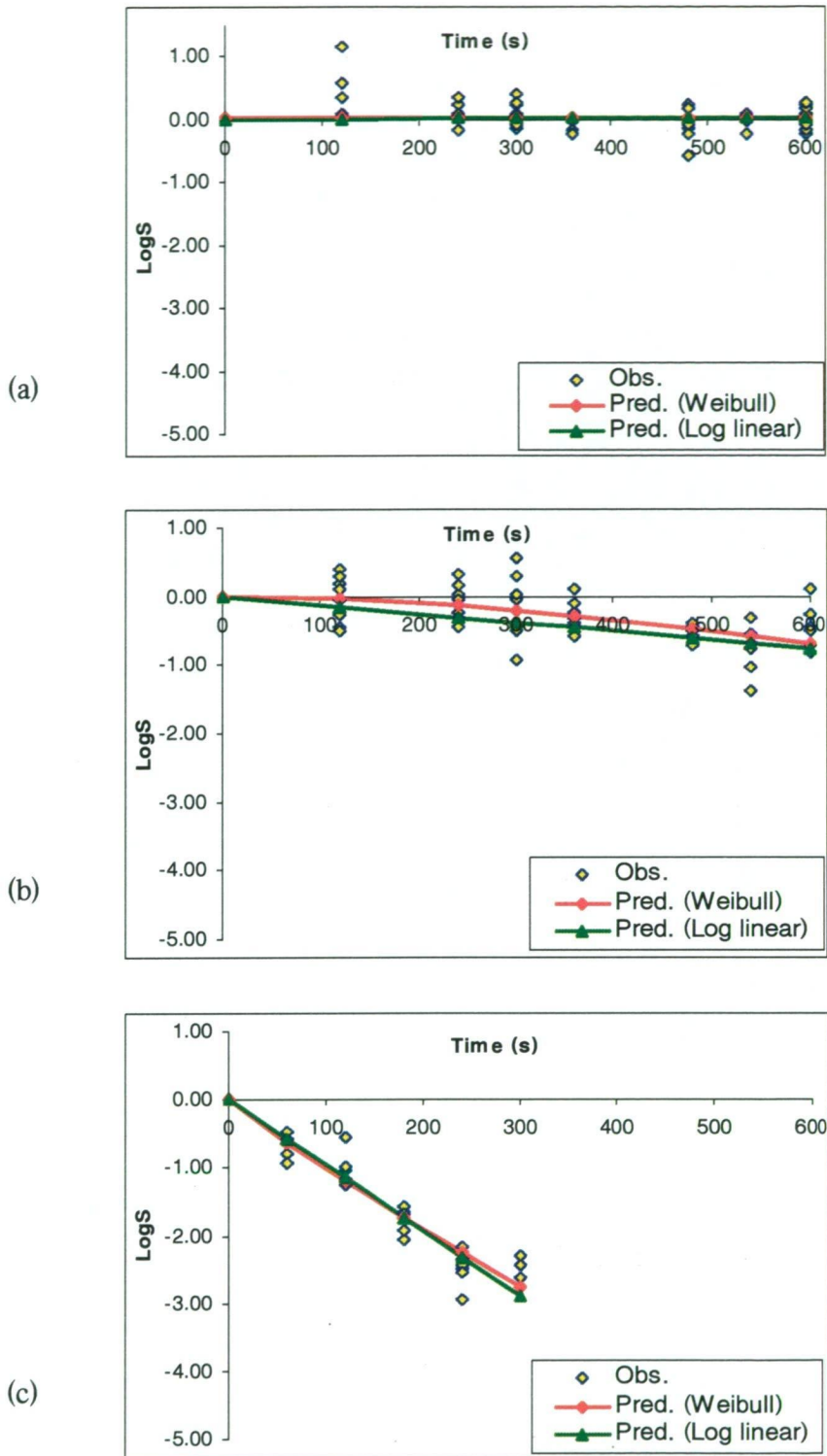


Figure 6.3. LogS values of hepatitis A virus predicted by the individual Weibull models (Weibull) and Equation 7 (Log linear), in comparison with the experimentally-derived LogS values (Obs.) for treatments with (a) 300 MPa, (b) 400 MPa and (c) 500 MPa in buffered media containing 30 ppt salt.

Table 6.1. Values for b and n in the Weibull curves fitted to the observed high pressure inactivation data presented in Figures 6.2 and 6.3, in relationship to salt concentration and pressure levels.

Salinity (ppt)	Pressure (MPa)		
	300	400	500
Fitted b values			
15	0.22	0.27	0.76
30	-0.031	0.0010	0.015
Fitted n values			
15	0.27	0.31	0.24
30	0.0018	1.7	0.91

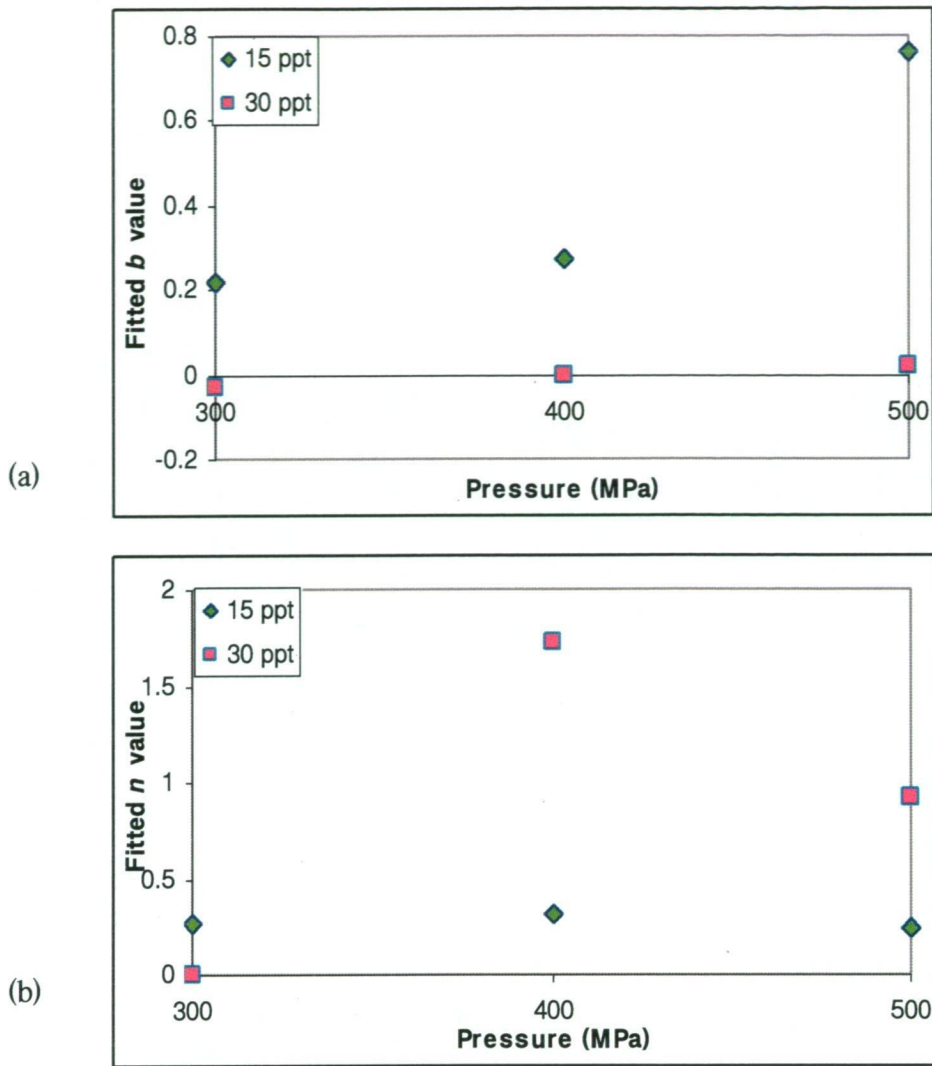


Figure 6.4. Relationship of (a) b and (b) n values in the Weibull curves fitted to the observed high pressure inactivation data presented in Figures 6.2 and 6.3, to levels of pressure and salt concentration of buffered media. The magnitude with which the values for b and n change in relationship to pressure level is erratic for both levels of salt concentration.

On the basis of the unpredictable relationship between values of b and n with salt concentration and pressure, further examination of model fitting capacity was performed only on the log-linear model. One such examination was to plot experimentally-observed LogS values against the LogS values predicted by the model (Figure 6.5). Data closer to the diagonal line (line of equivalence) indicate a better model, as do data distributed evenly on either side of the line (including each sub-set of data). Visual inspection of the graph indicates a reasonably even spread of data around the line of equivalence, with the magnitude of distribution around the line increasing with decreasing LogS values.

The log-linear model was also evaluated by sum of squared residuals (SS), coefficient of determination (R^2), bias factor (B_f) and accuracy factor (A_f) (Table 6.2). The model is generally fail-safe, as indicated by a bias factor <1.0 . The inherent variation that was observed in HPP inactivation data, particularly at 15 ppt salt, is reflected in the R^2 value of 0.613. Evaluation of the model was then performed against novel data, i.e. treatments in spiked oyster homogenate.

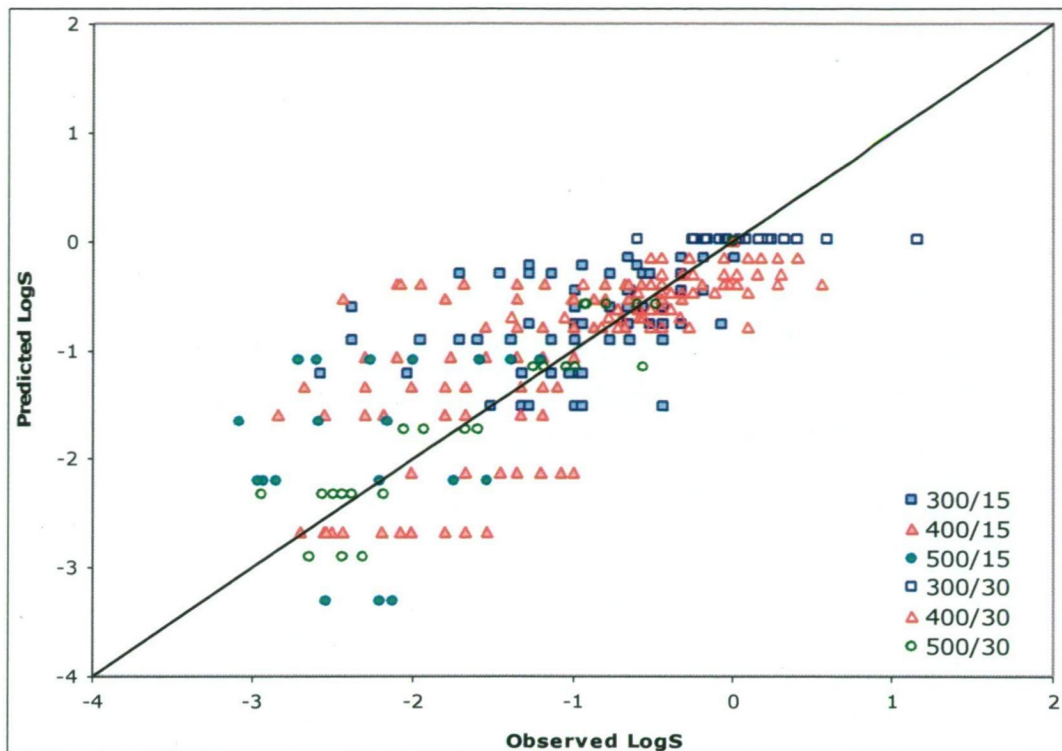


Figure 6.5. Plot of observed LogS values vs. LogS values predicted by the log-linear model for high pressure treatment of hepatitis A virus in buffered media.

Table 6.2. Evaluation of the fit of the log-linear model to observed high pressure processing inactivation data obtained from treatments of hepatitis A virus in buffered media. SS: Sum of squared residuals; R^2 : Coefficient of determination; B_f : Bias factor; A_f : Accuracy factor.

SS	117.9
B_f	0.794
A_f	2.51
R^2	0.613

6.3.3 Model validation in spiked oyster homogenate

Heat-sealed pouches were used for packaging spiked homogenate samples for HPP, whereas screw-capped cryotubes were used for experiments undertaken in buffered media. Whilst it may be possible for food packaging to affect the transfer of pressure into foods and therefore affect the extent of microbial inactivation, the flexible nature of both packaging materials in this study meant that any difference in the transmission of pressure through the two materials would be negligible (C. Stewart, personal communication).

Survival curves of individual LogS values predicted by the log-linear model in model validation trials at 15 ppt salt (Figure 6.6) and 30 ppt salt (Figure 6.7) were plotted. The results are in agreement with those from treatments in buffered media, that HAV is more sensitive to HPP at lower salt concentrations.

Observations of predicted curves indicate the model is generally 'fail-safe', underpredicting the magnitude of inactivation in oyster homogenate observed from treatments at 375 MPa and higher, while at 325 MPa and 15 ppt, the model is 'fail-dangerous', overpredicting the magnitude of inactivation observed in the treatments.

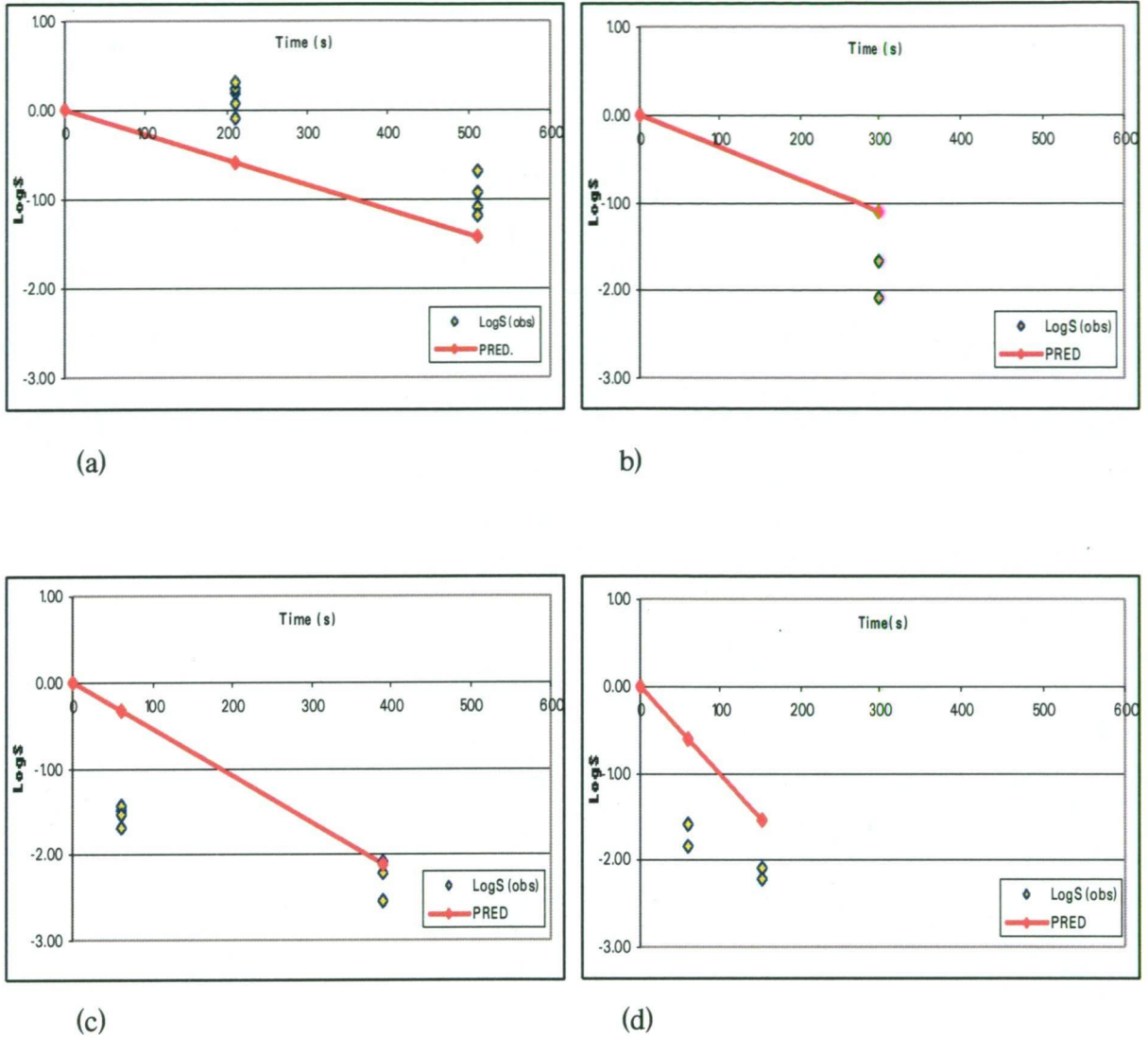


Figure 6.6. Log-linear survival curves of hepatitis A virus (HAV) resulting from high pressure treatments at (a) 325 MPa, (b) 375 MPa, (c) 425 MPa and (d) 475 MPa for model validation in oyster homogenate containing 15 ppt salt. No HAV was detected after 300 s treatment with 450 MPa, thus no curve is shown.

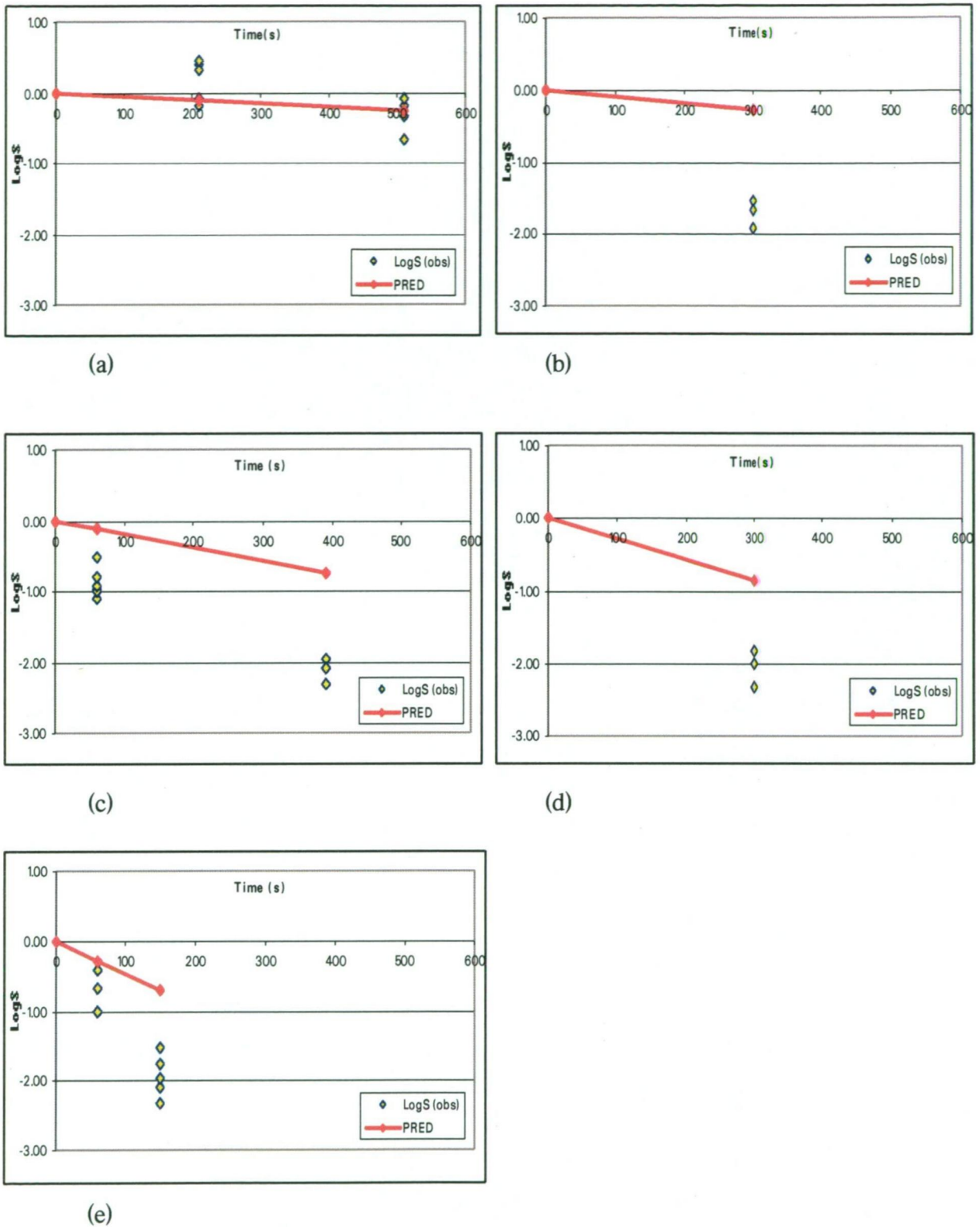


Figure 6.7. Log-linear survival curves of hepatitis A virus (HAV) resulting from high pressure treatments at (a) 325 MPa, (b) 375 MPa, (c) 425 MPa, (d) 450 MPa and (e) 475 MPa for model validation in oyster homogenate containing 30 ppt salt.

A graph of observed LogS values vs. predicted LogS values supports the observation that the model tends to underpredict inactivation in oyster homogenate (Figure 6.8). Further tests of the model's predictions in oyster homogenate demonstrate that the model's fitting capacity for oyster homogenate data is lower than that for buffered sample data (Table 6.3). In particular the R^2 value of 0.372 is low, indicating the difficulty in describing the variation within the data by the model.

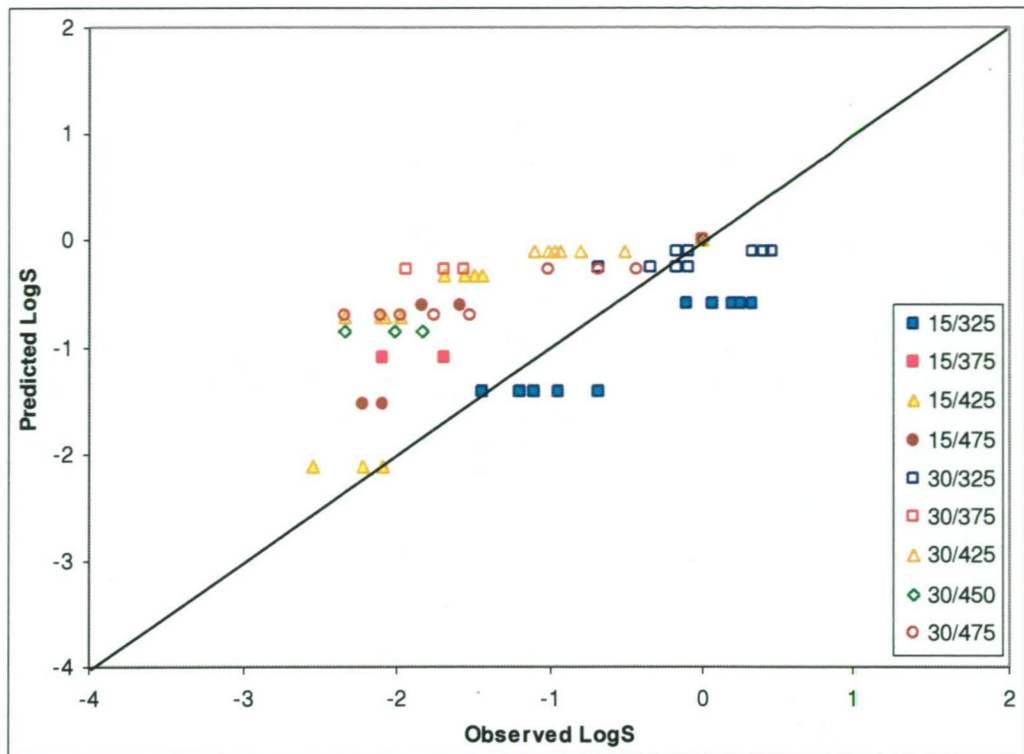


Figure 6.8. Plot of observed LogS vs. LogS predicted by the log linear model, from high pressure treatments of hepatitis A virus in oyster homogenate.

Table 6.3. Evaluation of the fit of the log-linear model to observed high pressure inactivation data observed from treatments of hepatitis A virus in oyster homogenate. SS: Sum of squared residuals; R^2 : Coefficient of determination; B_f : Bias factor; A_f : Accuracy factor.

SS	55.24
B_f	0.384
A_f	4.42
R^2	0.372

6.4 DISCUSSION

HAV was observed to be more sensitive to high pressure at a lower salinity, both in buffered media as well as in oyster homogenate, which is in agreement to the observations of other researchers. Kingsley *et al.* (2002) compared HPP inactivation of HAV in isotonic (4.1 ppt) growth media (DMEM with 10% FBS) with inactivation in growth media diluted 1:10 in 30 ppt seawater (final salinity 27.4 ppt). Higher pressures were required to inactivate HAV at high salinity within 5 min to an amount equivalent in isotonic media. For example, HAV was inactivated by approximately 7- \log_{10} PFU/ml following 5 min treatment with 450 MPa at ambient temperature. When diluted in seawater, however, HAV was inactivated by approximately 4- \log_{10} PFU/ml with the same treatment.

Smiddy *et al.* (2005) observed the protective effect of increased salt to high pressure inactivation of bacteria, when strains of *Vibrio mimicus*, *Escherichia coli* and *Listeria innocua*, suspended in buffered media containing 3.5% (35 ppt) or 0.5% (5 ppt) salt, were exposed to a range of pressures during 5 min treatments. Increased resistance to HPP was observed at the higher salt concentration by all bacteria tested. In fact, at pressures between 480 and 600 MPa, a 5- \log_{10} difference was observed in the level of high pressure-induced inactivation of all bacteria suspended in PBS compared to oyster homogenate.

A study by Calci *et al.* (2005) investigated the effect of 1 min high pressure treatments on HAV within contaminated shucked oysters. The Eastern oysters (*Crassostrea virginica*) used in that study were indigenous to seawater of between

5 and 30 ppt salinity, and were maintained in the laboratory in seawater ranging between 5 and 20 ppt. This variation of 15 ppt is large, and as the results in this study suggest, such a variation can affect the sensitivity of viruses dramatically. The salinity of harvest waters that oysters are grown in can vary dramatically between countries and growing areas due to rainfall or local estuarine environments, and is dependant on the marine environment, e.g. estuarine waters compared to coastal seawater. The salinity of Australian coastal waters generally remains between 30-37 ppt, although oyster farms located in estuarine environments may be subjected to a larger variation in salt concentration due to heavy rainfall and flooding or, conversely, drought conditions.

Salt is thought to stabilize microbial proteins by opposing enzyme unfolding and denaturation (Smiddy *et al.*, 2005). The intracellular ionic strength of oysters varies with that of their surrounding water. The results from this investigation showed that the salinity of the oyster harvest water may need to be taken into account when planning HPP regimes for the disinfection of oysters, due to the difference in HAV inactivation that may result. For the purpose of treating oysters with HPP to inactivate HAV or other pathogenic microorganisms, knowledge of the water salinity that oysters were harvested from prior to treatment will be useful to ensure sufficient processing. Alternatively a depuration process may be applied to adjust the ionic strength of oysters prior to HPP, and this could even be included into a marketing initiative to achieve a better-tasting product.

In several pressure and salt combinations HAV inactivation appeared to be higher in oyster homogenate than in buffered media. For example, HAV treated with 400 MPa for 300 s was inactivated by an average 1.79- \log_{10} and 0.18- \log_{10} TCID₅₀/ml in buffered media containing 15 ppt (Figure 6.2b) and 30 ppt (Figure 6.3b) salt, respectively. In the closest equivalent treatments in oyster homogenate adjusted to 15 ppt (Figure 6.6b) and 30 ppt (Figure 6.7b), HAV was inactivated by 1.98- \log_{10} and 1.72- \log_{10} TCID₅₀/ml, respectively, with 375 MPa for 300 s, i.e. with 25 MPa lower pressure than in buffered media.

Whole oysters were homogenized prior to artificial inoculation and pressure treatment. The homogenization disrupts oyster tissue and membranes, and probably releases cellular contents to expose the homogenized tissue to enzymatic degradation. This may have contributed to damage of viral capsid proteins in addition to the effects of high pressure, and increased the inactivation over virus samples in buffered media. In order to delay the spoilage of the homogenate and to reduce enzymatic activity, all samples were stored on ice or at 4°C, and virus extraction procedures were performed on the day after high pressure treatment.

Investigation may also be warranted into the role that oyster species may contribute to sensitivity of viruses to HPP, as differences in growth conditions, climate, metabolic rates and tissue structure may affect the distribution of virions within oyster tissue, and affect the protection afforded to virions from high pressure.

Smiddy *et al.* (2005) observed the protection conferred by oyster homogenate against high pressure to *V. mimicus*, *E. coli*, *L. innocua* and *L. monocytogenes*. In treatments that inactivated each microorganism suspended in PBS to levels less than the detection limit of the enumeration method, each bacterium was still able to be detected following the same treatment when suspended in oyster homogenate.

In the current study, model validation was performed by only altering the substrate in which HAV was suspended. The salt concentration in oyster homogenate was adjusted to that of the buffered media, while the HPP equipment and initial temperature of treatments remained constant between the initial data collection and the model validation. The results presented indicate the important influence that substrate composition has on the extent of microbial inactivation during HPP treatment.

A recent study investigating the sensitivity of murine norovirus (MNV-1) to high pressure reported the treatment of virus suspended in buffered media, as well as virus within naturally-contaminated shucked oysters, with various levels of high pressure (Kingsley *et al.*, 2007). Treatments were performed at 20°C for buffered samples and 5°C for oyster samples, and while the effect of substrate on sensitivity to high pressure appeared to be minimal in direct comparisons between treatments, these comparisons are not valid due to the different temperatures used. Studies in which these variables are tested should keep values constant to ensure accurate and meaningful comparisons can be made.

As already stated, Calci *et al.* (2005) observed the sensitivity of HAV to 1 min high pressure treatments within contaminated oysters. In this study, the authors compared the inactivation observed to the inactivation reported by Kingsley *et al.* (2002) in 5 min treatments on HAV suspended in buffered media. The reductions obtained at each pressure ($>1\text{-log}_{10}$, $>2\text{-log}_{10}$ and $>3\text{-log}_{10}$ PFU at 350, 375 and 400 MPa, respectively) were less in contaminated oysters than in buffered media, especially at higher pressures. Again, the authors could not conclude the substrate alone affected HAV inactivation, because different salinities, treatment times and starting temperatures were used in each study, and may all contribute to a different level of inactivation.

Treatment temperature has been shown on a number of occasions to affect virus inactivation by HPP. In a study by Chen *et al.* (2005), FCV was most resistant to high pressure at 20°C, with $<1\text{-log}_{10}$ reduction achieved after 4 min with 200 MPa, but pressure sensitivity was increased with higher and lower starting temperatures, with a 4.0 and 5.0 \log_{10} reduction from the same treatment at 50°C and -10°C. MNV-1 sensitivity to a 5 min, 350 MPa high pressure treatment was increased at temperatures lower than 20 and 30°C (Kingsley *et al.*, 2007), but HAV does not appear to share the same sensitivity characteristics. In 1 min treatments with 400 MPa, for instance, reductions of 1.0, 2.5, and 4.7- \log_{10} PFU/ml were observed at -10, 20 and 50°C (Kingsley *et al.*, 2006). Treatments with oysters at refrigerated temperatures could not be achieved with the high pressure unit used in the current study.

The aim of this study was to develop a HPP predictive inactivation model that can predict the inactivation of HAV in contaminated oyster tissue by interpolation between 300 and 500 MPa over a range of treatment times relevant to shellfish processing, without the need for further laboratory challenge testing. The Weibull model described HAV inactivation in buffered media well. The log-linear model also described the data well in 30 ppt buffer, but the variation between replicate data points and curvature of the inactivation meant in 15 ppt buffer its predictions were less accurate. The log-linear model, however, could be developed into a secondary model with greater confidence than the Weibull model. The individual Weibull models required 12 independent variables (each combination of pressure and salt concentration) compared to the two (pressure and salt concentration) employed in the log-linear model, and the relationships between these parameters were unpredictable. Non-linear curves have been used previously by a number of researchers to describe the microbial inactivation kinetics of pressure treatment over time, while log-linear curves have been used to plot the inactivation of HAV as a function of pressure, but not time (Kingsley *et al.*, 2002 and 2007; Calci *et al.*, 2005; Chen *et al.*, 2005).

The shapes of the Weibull curves (determined by the value for n) followed a pattern with increasing pressure that is difficult to predict and interpolate; i.e. the value for n is highest at 400 MPa (the middle pressure tested) at each salt concentration. The individual Weibull curves may be used separately to predict HAV inactivation under those specific conditions of pressure, salt concentration and time, i.e. as primary models. Predictions of inactivation at pressures between 300 and 500 MPa by interpolation, i.e. as a secondary model, cannot be achieved

using the Weibull function, and the log-linear model developed is most suitable. However, more challenge testing is required at pressures between those tested in this study to gain more data, in an effort to better understand the curvature during HAV inactivation, and in the case of the Weibull model, to gain a better understanding of the pattern of n values in respect to salt concentration and pressure.

The results presented in this chapter confirm the sensitivity of HAV to high pressure, with increased sensitivity observed at a lower salt concentration. For most treatments higher than 325 MPa, HAV suspended in oyster homogenate was inactivated by a greater amount than in buffered media, and therefore was underpredicted by the log-linear model in the treatments with oyster homogenate, giving a fail-safe prediction. The log-linear model described in equation 6 may be a useful aid for Australian shellfish processors wishing to implement HPP into an oyster processing regime to increase the microbial safety of the product. The collection of more inactivation data between the pressures studied in this chapter is desirable so that further adjustments can be made to improve the accuracy of the model's predictions.

Chapter 7: Rapid and quantitative detection of hepatitis A virus by quantitative real-time reverse-transcription PCR

7.1 INTRODUCTION

HAV is a difficult virus to propagate in the laboratory, and while plaque-forming cell culture-adapted isolates are commonly used in challenge studies, environmental isolates of HAV remain difficult to grow in cell culture, and may not form plaques in cells during infectivity assays (Konduru and Kaplan, 2006). The difficulty of HAV detection and quantification in cell culture has therefore given rise to the popularity of molecular biological techniques, such as quantitative reverse transcription (RT) real-time polymerase chain reaction (qRT-PCR).

Quantitative RT-PCR has the potential for increased sensitivity and specificity over the traditional cell culture infectivity assays for virus detection, and like traditional RT-PCR, allows detection of small numbers of viral genome in environmental or clinical samples in a shorter time than traditional cell culture methods.

A barrier to the sensitive detection of viruses using molecular techniques is the potential presence of substances that inhibit PCR, such as heavy metals, glycogen and humic acids in foods and/or environmental samples. A number of methods

have been described in the literature for separating virions or viral RNA from such inhibitory substances prior to PCR to ensure detection of the specific target (Atmar *et al.*, 1993; Lopez-Sabater *et al.*, 1997; Dix and Jaykus, 1998; Kingsley and Richards, 2001; Shan *et al.*, 2005; Hewitt and Greening, 2006). One such separation method is immuno-magnetic separation (IMS), where monoclonal antibodies against the target virus are bound to micron-sized magnetic beads and added to a sample. The target virus or antigen is bound to the antibody on the beads, and then the whole complex can be separated from the suspending medium using a magnet. A series of washing steps can further purify the target from PCR inhibitors prior to the elution of the target from beads.

The qRT-PCR assay described in Chapter 5 detected HAV RNA in contaminated oysters when the virus was undetectable by tissue culture infectivity assay. The work described in this chapter details the study undertaken to improve the sensitivity of the assay, by incorporating IMS purification into the virus recovery procedure as an additional purification and concentration step, particularly as a means of removal of PCR inhibitors.

Several methods have been described which incorporate an IMS purification technique prior to detection of viruses in food or environmental samples using RT-PCR techniques (Deng *et al.*, 1994; Lopez-Sabater *et al.*, 1997; Shan *et al.*, 2005). The use of IMS offers sample purification from inhibitory substances, and target virus concentration by reducing the volume of eluent. This chapter describes the development of a method to detect and quantify HAV from oyster homogenate using qRT-PCR after additional purification with IMS.

7.2 METHODS

7.2.1 Quantitative RT-PCR

Quantitative RT-PCR was performed as described in Chapter 5, section 5.2.6.

Two primer sets were chosen to evaluate their amplification ability in the qRT-PCR assay (Table 7.1). The primer pair HAVF and HAVR were originally described by Robertson *et al.* (1992) during an investigation to determine the genetic relatedness of 152 unique wild-type and cell culture strains of HAV, and has since been used during RT-PCR for HAV detection from oysters (Kingsley and Richards, 2001). The second primer pair, JotFor and JotRev, were described by Jothikumar *et al.* (2005), in a protocol to develop a TaqMan qRT-PCR assay for rapid screening of HAV in clinical and environmental samples. Several concentrations of each primer pair were tested in the reaction mix with dilutions of RNA extracted from buffered HAV stock. Amplification curves and melt curves were subsequently analysed for non-specific amplification, Ct values and dissociation peaks.

Table 7.1. Primers evaluated for amplification of HAV RNA in qRT-PCR.

T_M denotes melting temperature, bp denotes base pair.

Name	Sequence (5'-3')	T_M (°C)	Amplicon length (bp)	Reference
JotFor	GGTAGGCTACGGGTGAAAC	60	89	Jothikumar <i>et al.</i> (2005)
JotRev	AACAACTCACCAATATCCGC	58		
HAVF	TATTTGTCTGTCACAGAACATCAG	49	267	Robertson <i>et al.</i> (1992)
HAVR	AGGAGGTGGAAGCACTTCATTGA	58		

7.2.2 HAV dilution series and standard curve

A duplicate 10-fold HAV dilution series was prepared in sterile PBS from 1:1 to 1:100,000. Each duplicate dilution was quantified by a triplicate infectivity assay, and the average of the six replicate assays was used in all subsequent titre calculations. RNA was extracted from HAV dilutions and a qRT-PCR performed as described above to generate the standard curve of Ct vs. \log_{10} starting quantity, which in this case is the corresponding infectious HAV titre of the RNA within the reaction. The titre of infectious HAV in a sample can be calculated by marking its Ct value on the standard curve.

7.2.3 RNA extraction

RNA was extracted from each dilution by two methods; a heat lysis method that releases RNA directly within the reaction tube without further purification, and an RNA purification method using the Qiagen QIAamp[®] Viral RNA kit (Qiagen method). The heat lysis method uses heat to disrupt viral capsids, theoretically giving a yield of virtually 100% of viral RNA within the sample. Due to the purification steps involved in the Qiagen method, performed as was described in Chapter 5, a higher chance exists of loss of RNA yield. A higher yield of purified RNA increases the range of the qRT-PCR standard curve, ensuring detection and quantification of lower amounts of virions.

In the Qiagen method, a 140 μ l aliquot of HAV was transferred to a microcentrifuge tube containing 560 μ l lysis buffer AVL supplied in the kit, and all other aspects of the protocol adhered to manufacturer's instructions. Purified RNA was eluted in 80 μ l RNase-free water containing 0.04% sodium azide (Qiagen).

In the heat lysis method, 50 μ l of each HAV dilution was transferred to a 0.2 ml PCR tube and heated to 99°C for 5 min in a thermocycler (Applied Biosystems, U.S.A.). Samples were then transferred to ice for at least 5 min prior to storage at -80°C.

7.2.4 Artificial inoculation of oyster samples with HAV

Ten grams of oyster homogenate was decanted into disposable sterile centrifuge tubes, and inoculated with 0.5 ml of the HAV dilutions described in 7.2.2, between the 1:100 (1.04×10^4 TCID₅₀/ml) and 1:100,000 (2.98 TCID₅₀/ml) dilutions. A negative control was also included, where sterile PBS was substituted for HAV.

Samples were mixed by inversion and incubated on ice for 30 min to promote adsorption of HAV to homogenate. To then elute virions, inoculated homogenate was diluted 1:4 (w/v) in glycine buffer, mixed vigorously, and incubated at room temperature for 15 min. After centrifugation at 15,000 \times g for 1 h at 4°C, the pH of the supernatant was adjusted to 7.0 \pm 0.3 pH units with 1 M HCl, treated with a 1:50 dilution of antibiotic/antimycotic solution, and virions were purified by IMS purification prior to RNA extraction with the Qiagen method.

7.2.5 Immunomagnetic separation

Dynabeads® Pan Mouse IgG (DynaL Biotech, Germany) were used for IMS purification. Beads were coated with 0.5 µg anti-HAV monoclonal antibody per 10^7 beads by incubating beads and antibody with rotation for 1 h at 4°C according to the manufacturer's instructions. Coated beads were then collected with a magnet, separated from the supernatant, washed with sterile-filtered PBS with 0.1% bovine albumin (pH 7.4), and resuspended for virus capture.

One millilitre of recovered HAV was added to 10^7 prepared beads for capture in a microcentrifuge tube. For a negative control, 1ml PBS was substituted for HAV. Samples were incubated with gentle mixing by rotation on an automated rotator at 4°C for 30 min, then collected with a magnet and supernatant discarded. Beads were washed 4 times, and then resuspended in 50 µl RNase-free water. RNA was released from virions by heat lysis, and immediately treated with 2 µl RNase inhibitor (RNasin® Plus; Promega Corp., U.S.A.) prior to qRT-PCR.

7.2.6 Qiagen method

RNA was extracted from a 140 µl aliquot of extracted HAV by the Qiagen method as described in 7.2.3.

7.3 RESULTS

7.3.1 Primer optimisation

SYBR Green is a chelating dye that fluoresces when bound to any double stranded DNA; it is not sequence-specific. Therefore, confirmation that the PCR product is the target of interest is possible by melt curve analysis. A dissociation peak is observed at an amplicon's T_M , and is representative of the sudden drop in fluorescence observed when each strand of the DNA amplicon comes apart.

Melt curves were generated to evaluate the JotFor/JotRev and HAVF/HAVR primer sets for their amplification ability in the qRT-PCR assay (Figure 7.1). At a concentration of 0.3 μM per reaction, JotFor/JotRev amplified an 89 bp amplicon with a T_M of $78 \pm 1.0^\circ\text{C}$ (Figure 7.1a). A single dissociation peak was observed at both dilutions of RNA and with each primer concentration tested, with negligible non-specific amplification. In contrast, the HAVF/HAVR primer set amplified non-specific products with a T_M of $72\text{--}73^\circ\text{C}$ at low concentrations of HAV RNA in the reaction, and also in the no template control when no HAV RNA was present (Figure 7.1b). The T_M of the HAV amplicon produced by the HAVF/HAVR primer set was $80^\circ\text{C} \pm 1.0^\circ\text{C}$. The JotFor/JotRev primer set was consequently used in subsequent qRT-PCR assays, at a concentration of 0.3 μM per reaction.

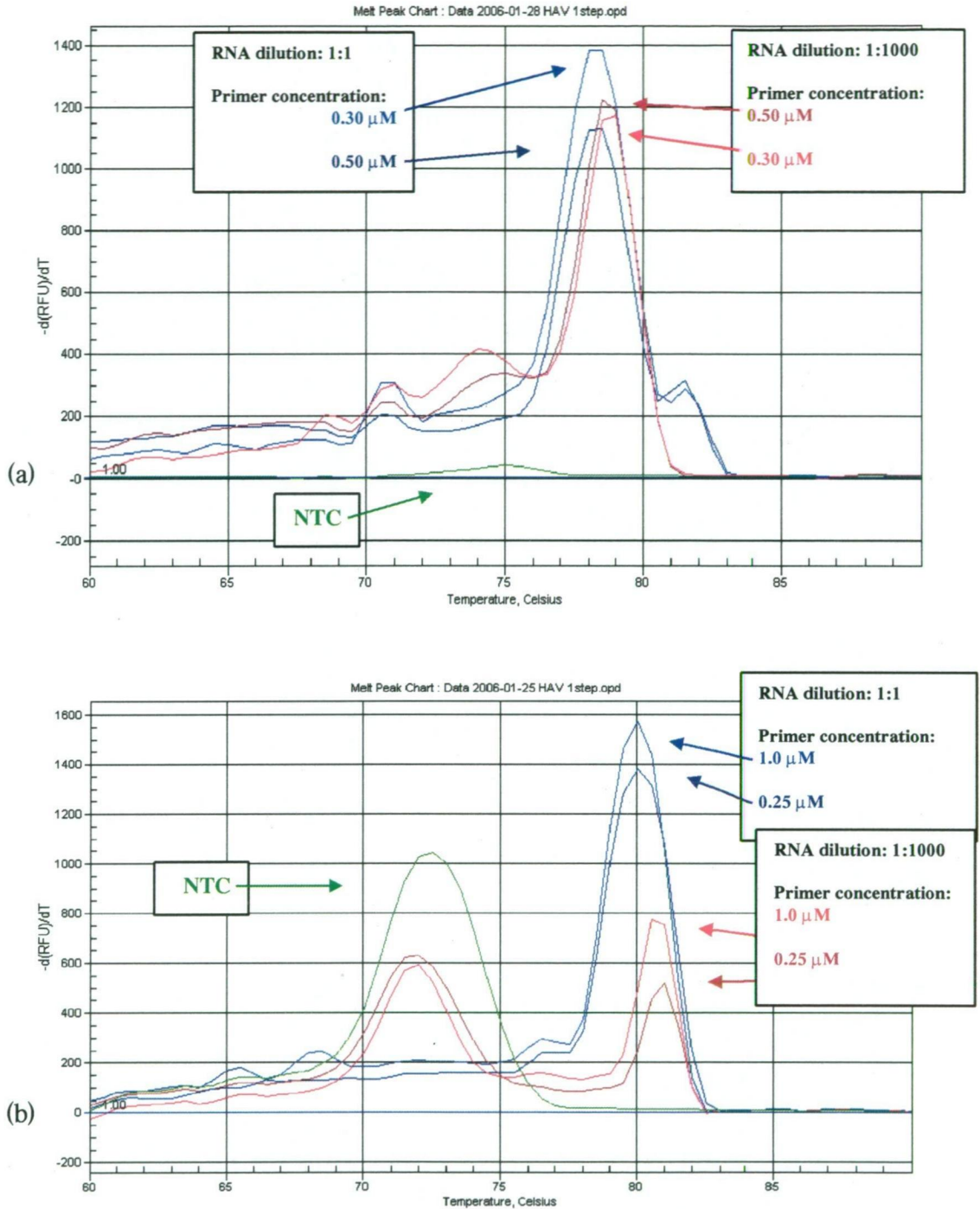


Figure 7.1. Melt curve analysis following qRT-PCR amplification of hepatitis A virus (HAV) RNA dilutions. Expected dissociation peak of amplicon using JotFor/JotRev: $78 \pm 1.0^\circ\text{C}$ (a), and using HAVF/HAVR: $80 \pm 1.0^\circ\text{C}$ (b). NTC denotes no template control.

7.3.2 Standard curve

Duplicate 10-fold serial dilutions of HAV were prepared in sterile PBS to generate a qRT-PCR standard curve. The average HAV titre in each dilution was determined by triplicate infectivity assays between the 1:1 (1.54×10^6 TCID₅₀/ml) and 1:10,000 dilutions (2.98 TCID₅₀/ml), but the titre of HAV in the 1:100,000 dilution was below the quantification limit of the infectivity assay (29.5 TCID₅₀/ml) and was therefore assigned a nominal titre of 2.98 TCID₅₀/ml, i.e. 1- \log_{10} below the infectious titre of the 1:10,000 dilution (Table 7.2). The standard curve loses linearity with any higher dilutions after 1:100,000, so the lower quantification limit of the qRT-PCR assay is therefore 2.98 TCID₅₀/ml.

The infectious titre of each dilution (TCID₅₀/ml) was converted to the \log_{10} infectious titre within the qRT-PCR reaction. A linear relationship was observed between infectious HAV titre and Ct value from RNA extracted by the Qiagen method, with the coefficient of determination (R^2) >0.995 between the neat and 1:100,000 dilutions (Figure 7.2). Considerable variation was seen from the trend line in the standard curve developed from heat-lysed RNA ($R^2 = 0.929$). No purification step is involved during the heat-lysis procedure, and as such contaminants may be present in the RNA sample that may interfere with qRT-PCR. Due to the high R^2 and better fit to the trend line, the standard curve from Qiagen-extracted RNA was repeated in four separate qRT-PCR assays. The HAV titre of each dilution, in TCID₅₀/ml, was converted to the \log_{10} infectious HAV titre used in the qRT-PCR reaction (TCID₅₀/reaction), and average Ct values from each dilution in five replicate runs were used in subsequent analyses (Figure 7.3).

Table 7.2. Infectious titre of hepatitis A virus (HAV) at each 10-fold dilution in the standard curve. Titres are the average of duplicate dilution series, each assayed in triplicate, except 1:100,000, which is below quantification limit of infectivity assay.

Dilution	Average HAV titre (TCID₅₀/ml)
1:1	1.54×10^6
1:10	1.77×10^5
1:100	1.04×10^4
1:1000	2.75×10^2
1:10,000	2.98×10^1
1:100,000	2.98×10^0

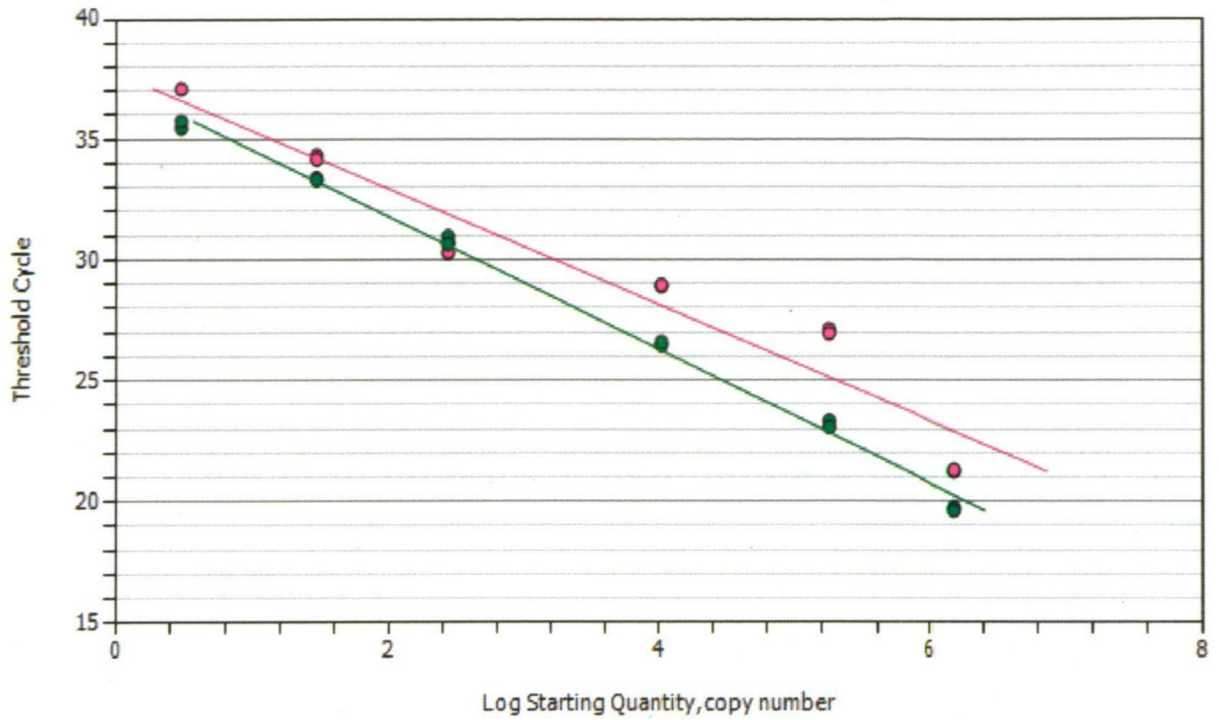


Figure 7.2. Standard curve of Ct vs. \log_{10} infectious hepatitis A virus (HAV) titre within the reaction. Green points are Ct values from RNA extracted by Qiagen method; line of best fit R^2 : 0.996; slope: -2.754; Y-intercept: 37.232. Pink points are Ct values from RNA extracted by heat lysis; line of best fit R^2 : 0.929; slope: -2.398; Y-intercept: 37.730.

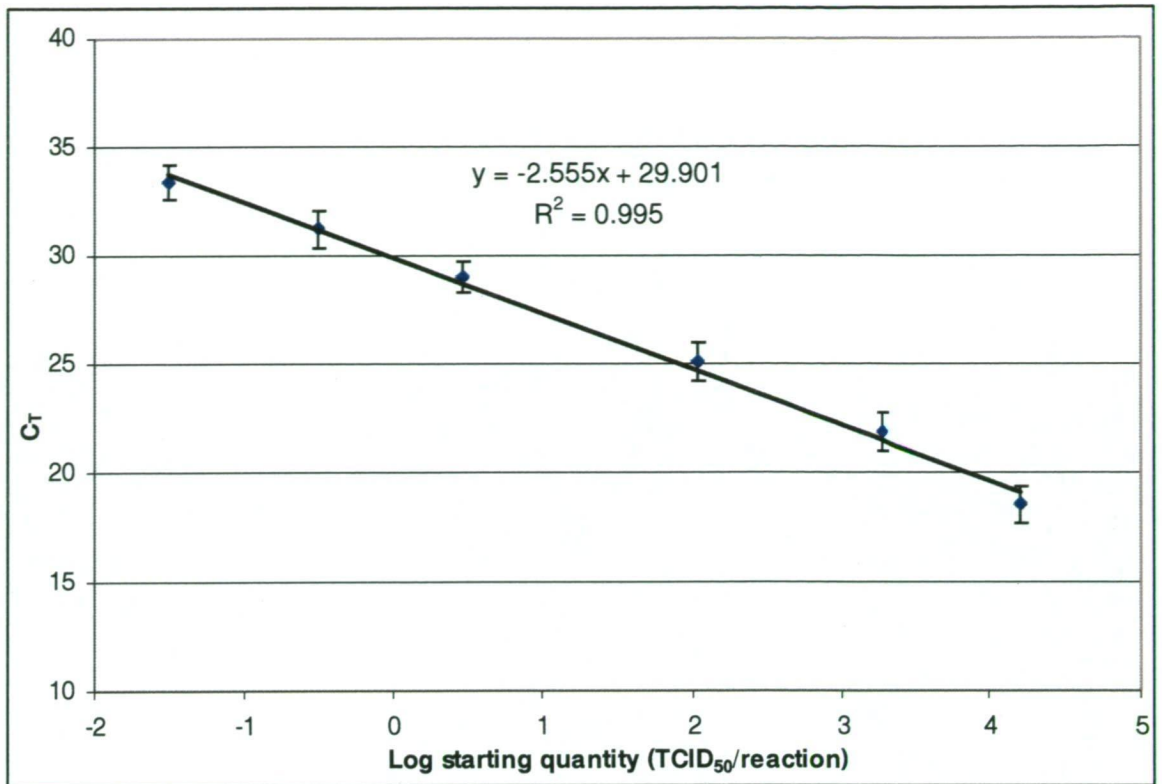
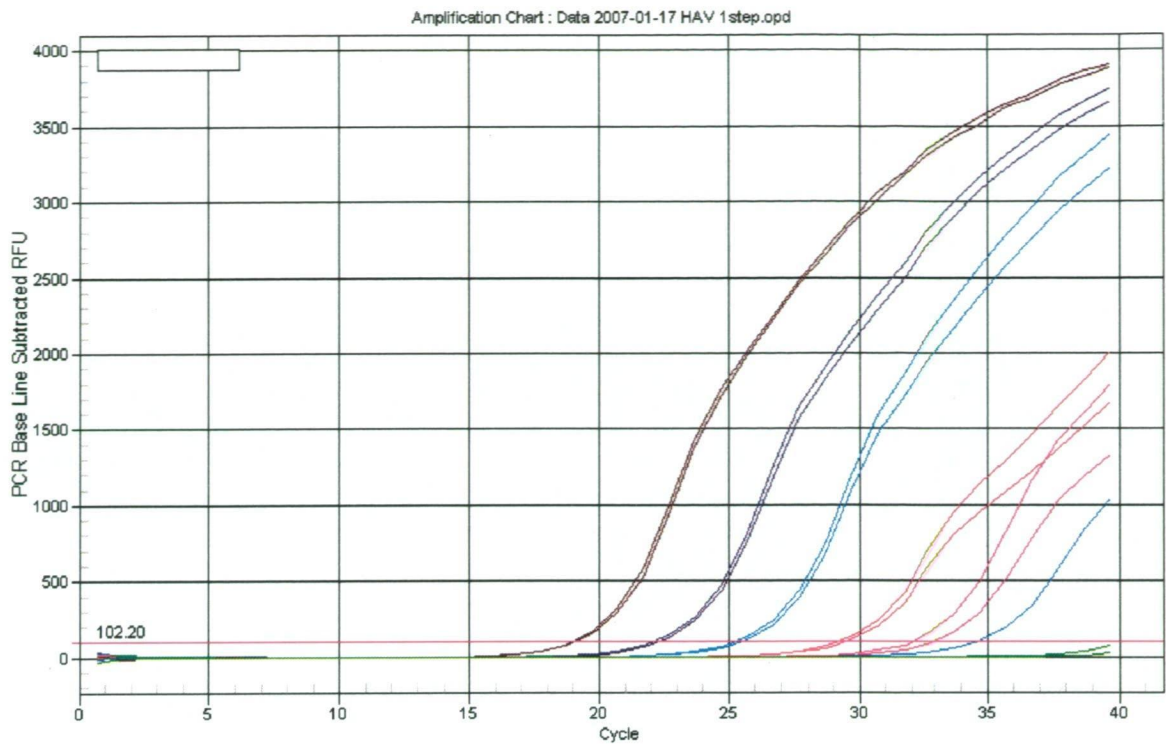


Figure 7.3. Quantitative RT-PCR standard curve using RNA extracted by the Qiagen method. Each point is the average cycle threshold (Ct) of five replicate assays, and error bars denote standard deviation (SD) of the five replicates. The difference between the maximum and minimum Ct at each dilution is listed (Range).

The amplification plot from one of the replicate qRT-PCR assays for HAV standards provides a graphical representation of the target RNA sequence being amplified, by measuring relative fluorescence units (RFU) over the length of the reaction (cycles) (Figure 7.4). The Ct value is defined as the cycle at which a designated RFU value (threshold; indicated by pink horizontal line in Figure 7.4) is intersected during amplification, and in this curve is derived by the Bio-Rad iQ5 software to intersect the curves during log-linear amplification.

Confirmation of HAV amplification is achieved by examination of the melt curve for the expected 89bp HAV amplicon dissociation peak of $78.0 \pm 1.0^{\circ}\text{C}$ (Figure 7.5). It can be seen from the melt curve that HAV amplicon was generated, and negligible non-specific amplification was observed during qRT-PCR.



Dilution	Average Ct
1:1	19.00
1:10	22.22
1:100	25.37
1:1000	29.38
1:10,000	32.47
1:100,000	34.69

Figure 7.4. Quantitative RT-PCR amplification curve of hepatitis A virus standard 10-fold dilution series. Threshold (Pink horizontal line) indicates Ct value for each reaction. Each dilution in duplicate, except 1:100,000; a single assay. The no template control (NTC) is indicated by a green line, and did not cross the threshold.

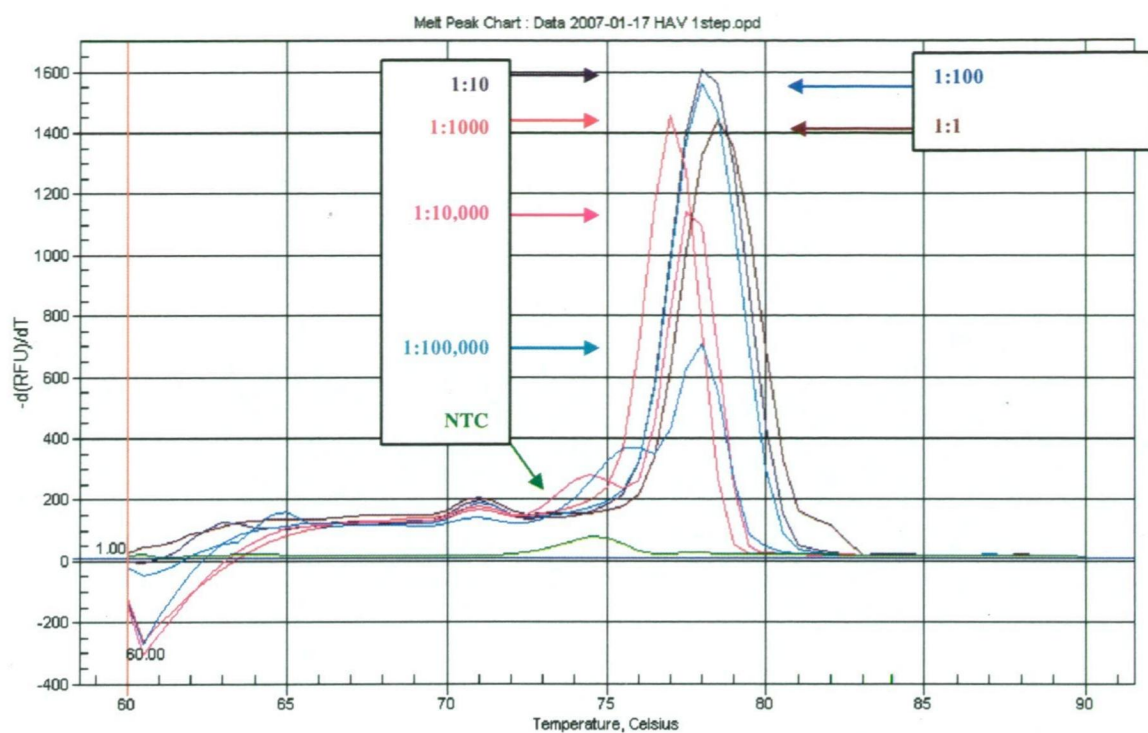


Figure 7.5. Melt curve of qRT-PCR amplification of hepatitis A virus (HAV) standard 10-fold dilution series. Expected dissociation peak of the 89bp HAV amplicon: 78.0°C \pm 1.0°C.

7.3.3 Detection and quantification of hepatitis A virus in spiked oyster homogenate

Ten gram samples of oyster homogenate were spiked with 10-fold serial dilutions of HAV from the 1:100 dilution to 1:100,000 dilution listed in Table 7.2. The titre of HAV within each 10 g oyster homogenate sample was 5200, 138, 14.9, and approximately 1.49 TCID₅₀.

RNA extracted from HAV following purification from oyster homogenate by the Qiagen method could not be detected following qRT-PCR. No amplification of the target sequence was detected, and smears were observed in the subsequent electrophoresis gel (Figure 7.6). The smears, indicating the presence of degraded nucleic acid in the reactions, were also present in the negative oyster control, in which HAV inoculum was replaced with PBS, but was not present in the negative control in which sterile PBS only was processed by the Qiagen method. This indicates that inhibitors were introduced into the qRT-PCR reaction from the oyster tissue due to inefficient removal during RNA purification. In an effort to dilute the inhibitory substances out, 1:10 and 1:100 dilutions of the HAV RNA were made in nuclease-free water. This is a simple technique that can be used to overcome inhibition of PCR, and while better-defined peaks were observed in the melt curve analysis as a result, they were not at the expected melting temperature for HAV, and often there were more than three peaks in the one sample. This indicates the amplification of non-specific RNA but not HAV RNA, meaning there may not have been a detectable amount of HAV RNA in the sample for detection by qRT-PCR.

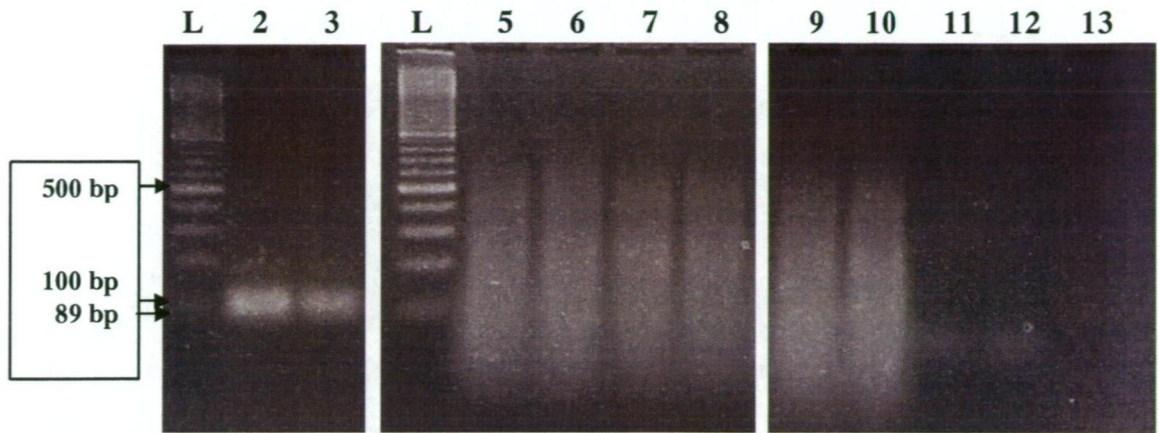


Figure 7.6. Hepatitis A virus (HAV) qRT-PCR products following RNA extraction by the Qiagen method from HAV recovered from oyster homogenate. Each segment is from a single gel. Lane L: 100 bp DNA ladder; lanes 2 and 3: HAV buffered stock, with RNA extracted by Qiagen method. Lanes 5 to 8: inoculation with 5.20×10^3 TCID₅₀ (higher dilutions gave smears of the same intensity). Lanes 9 and 10: non-inoculated oyster control; lanes 11 and 12: negative control, with Qiagen method performed on sterile PBS (no oyster homogenate). Lane 13: blank.

HAV RNA was detected by qRT-PCR in samples recovered from oyster homogenate with the crude virus extraction technique described in chapter 4 and IMS, in five out of eight (63%) samples inoculated with 5.20×10^3 TCID₅₀/10 g, and in one out of eight (12.5%) samples inoculated with 1.38×10^2 TCID₅₀/10 g (Table 7.3). This corresponds to 1.25×10^1 and 3.31×10^{-1} TCID₅₀/qRT-PCR reaction, respectively, and unlike the RNA extracted by the Qiagen method for these samples, defined and specific bands were visible following gel electrophoresis. The electrophoresis gel in Figure 7.7 shows that three of the four replicate samples tested are positive for HAV RNA.

Confirmation of amplification of the specific region of interest was achieved by examining together banding on the electrophoresis gel and dissociation peaks on the melt curve. In Figure 7.8, the melt curve confirms that HAV was detected in three out of four samples from oyster homogenate, but the dissociation peak of the fourth sample at 74°C is indicative of non-specific amplification. The non-template control (NTC), where sterile PBS replaced HAV in oyster homogenate, can also be identified in the melt curve, with a low, wide peak indicative of degraded nucleic acid within the reaction and seen as a smear during electrophoresis.

Table 7.3. Results of IMS and heat lysis for purification and extraction of hepatitis A virus (HAV) RNA from artificially inoculated oyster homogenate.

Titre of HAV in oyster	Expected HAV titre in reaction	Samples positive for HAV by qRT-PCR	
log₁₀ TCID₅₀/10g	log₁₀ TCID₅₀/reaction*	IMS	Qiagen
3.72	1.10	5/8	0/4
2.14	-0.48	1/8	0/4
1.17	-1.46	0/8	0/4
0.17	-2.46	0/8	0/4

*log₁₀ TCID₅₀/reaction is the equivalent titre within the RNA added to the qRT-PCR reaction, assuming 100% of HAV inoculated in oyster homogenate was recovered.

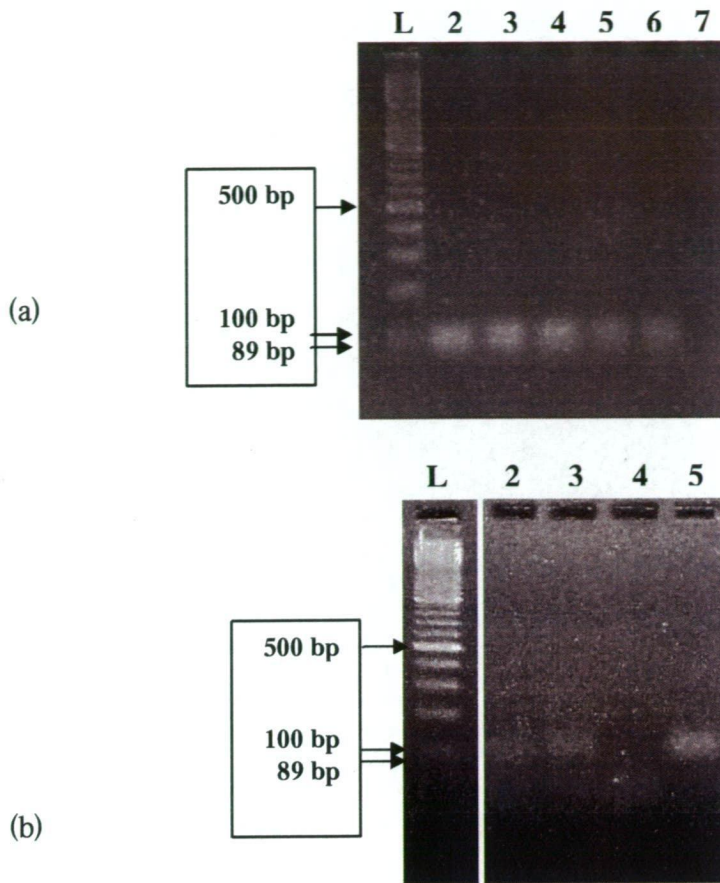


Figure 7.7. Hepatitis A virus (HAV) qRT-PCR products.

(a) HAV standard dilutions prior to inoculation into oyster homogenates.

Lane L: 100 bp DNA ladder; lane 2: 1.54×10^6 TCID₅₀/ml; lane 3: 1.77×10^5 TCID₅₀/ml; lane 4: 1.04×10^4 TCID₅₀/ml; lane 5: 2.75×10^2 TCID₅₀/ml; lane 6: 2.98×10^1 TCID₅₀/ml virus stock; lane 7: no template control. Expected HAV amplicon length: 89 bp.

(b) RNA extracted from HAV recovered by IMS from oyster homogenate.

Lane L: 100 bp DNA ladder; lanes 2-5: inoculation of 10g homogenate with 5.20×10^3 TCID₅₀.

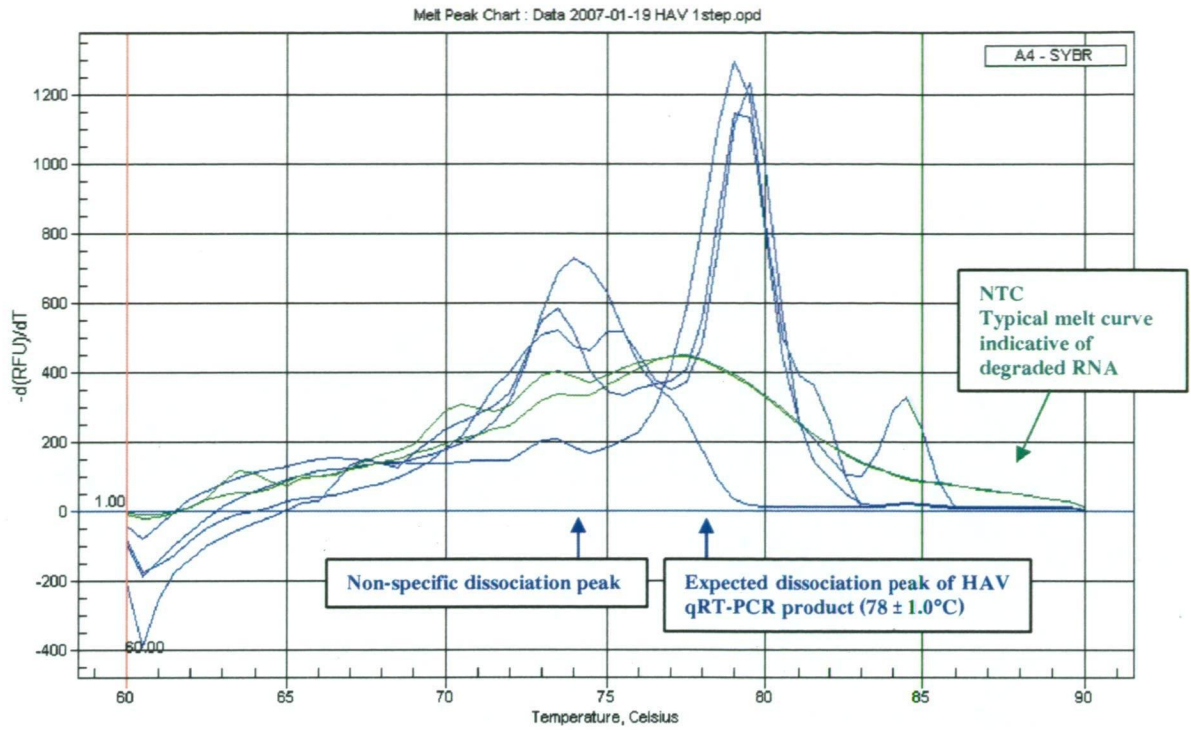


Figure 7.8. Melt curve confirmation of qRT-PCR amplification of hepatitis A virus (HAV) RNA from inoculated oyster homogenate. Three out of the four samples, coloured blue, are positive for HAV, as determined in the gel in Figure 7.7b.

The HAV RNA detected by qRT-PCR was below the quantification limit of the assay, i.e. outside of the range of the standard curve. The Ct range of the standard curve is from 18.55 to 33.36 cycles, yet the Ct values of samples detected from oyster homogenate ranged from 34 – 36 cycles, indicating the amount of HAV RNA detected in samples purified from oyster homogenate is below -1.50-log_{10} TCID₅₀/reaction (Figure 7.3). Table 7.3 lists the expected equivalent HAV titre in the qRT-PCR reaction, assuming 100% of HAV was recovered with the combined crude extraction and IMS recovery method. Had this been the case, the 3 lowest dilutions would have all contained $>1.5\text{-log}_{10}$ TCID₅₀ equivalent RNA in the reaction. As the results show, however, HAV could only be detected in one out eight replicates in the dilution containing -0.48-log_{10} TCID₅₀/reaction. PCR inhibitors were obviously active, and not completely removed by the virus recovery procedure, reducing the sensitivity of the assay as a result.

7.4 DISCUSSION

This chapter describes the development of a qRT-PCR assay to serve as an alternative method to the lengthy and laborious cell culture infectivity assay for detection and quantification of HAV in oyster meat. The assay was developed from that described in Chapter 5, which used a Qiagen method to extract RNA from virions recovered from oyster by the crude extraction method. That qRT-PCR method was more sensitive than conventional cell culture assay, detecting HAV RNA in contaminated oysters when virus was undetectable by infectivity assay.

This chapter described a method of purification that used IMS technology to separate virions from inhibitory substances by antibody capture prior to detection and quantification by qRT-PCR. Samples were concentrated 20-fold to improve detection. For comparison, the Qiagen method concentrated samples by less than 2-fold, and may have been a contributing factor to the inability to detect HAV RNA in oyster homogenate.

Figure 7.6 shows an electrophoresis gel in which 89-bp amplicons are visible for HAV buffered stock and no specific bands visible in the no template control, yet the samples of RNA extracted from virions recovered from oyster tissue cannot be differentiated from the smearing visible on the gel. The smearing is a visual representation of many different lengths of nucleic acid migrating through the gel, and may be caused by nucleic acid degradation. Ribonuclease (RNase and DNase) activity, which can be responsible for nucleic acid degradation, was

quenched upon addition of the lysis buffer AVL in the Qiagen method (results not shown), and no other samples suffered from degraded nucleic acid, indicating the issue was specific to the samples extracted from oyster. The column membrane used in the Qiagen method specifically binds RNA, although it cannot differentiate between RNA of different sources. Logically, RNA from several sources, such as oyster tissue, bacteria, microalgae, and even other viruses could be co-purified with HAV RNA during Qiagen extraction. The specificity of the primers used in qRT-PCR for the target nucleic acid is therefore critical in ensuring it and no other nucleic acid is amplified. In this study, inhibitory substances co-purified with target RNA and introduced into qRT-PCR may have interfered with the activity of the DNA polymerase or reverse transcriptase enzymes, causing the non-specific amplification, and helping to explain the smearing observed on the gel.

The removal of inhibitory substances that are present in foods and environmental samples has long been an issue to overcome when detecting viruses by PCR techniques. Purification procedures employed may not only remove inhibitors, but may also reduce the yield of viral RNA. A compromise may need to be reached, between a high yield of RNA and a highly purified sample. In the current study, the crude extraction method was used to initially recover virions from the oyster tissue prior to RNA extraction by each of the methods described. The crude extraction method was initially described by Kingsley and Richards (2003) as a method developed for simplicity, and was not effective in removing plaque assay inhibitors and cytotoxic substances. The methods for RNA purification in this study therefore needed to be effective in isolating viral RNA

from inhibitory substances. The Qiagen method uses a spin column containing a silica-gel membrane that binds RNA specifically while allowing inhibitors, including proteins, to pass through during wash steps. The load of inhibitors may have been too great in this study due to the lack of purification performed on the virus sample during crude extraction, and were passed through the filter with RNA during elution, affecting qRT-PCR. The IMS protocol, however, uses monoclonal antibodies specific to the viral capsid antigen, and contains many wash steps that may be more effective in removing the inhibitors prior to lysis of the capsid. Antibody-capture of intact HAV capsid antigen also provides a higher level of confidence, but still no assurance, that the RNA detected came from an undamaged, infectious virion.

While neither method is capable of differentiating RNA from infectious and non-infectious virions, use of IMS during virion purification ensures only antigen-captured virions are isolated for RNA extraction, meaning the epitope, determined by the surface protein structure of the virion capsid, and targeted by the monoclonal antibody, must be intact for antibody-antigen binding to occur. This does not guarantee that the genetic material detected in a sample will be from an infectious virion, but it eliminates detection of naked RNA in a sample, and increases the likelihood that virions detected are infectious.

The detection sensitivity of HAV from oyster homogenates using qRT-PCR assay was 138 (2.14-log₁₀) TCID₅₀/10g oyster homogenate. Further improvements to the virus recovery method can be envisaged that could improve sample purity prior to qRT-PCR, and greater sample concentration may increase the sensitivity of the assay. For example, the crude extraction method is simple to perform, but contains no concentration or purification steps.

Preliminary investigation determined that the method was ineffective in capturing virions from high-particulate samples, and that centrifugation of samples is required to pellet solid matter prior to IMS purification. Only 1 ml of the final volume following crude extraction is used for IMS purification, so there is scope to incorporate additional sample purification and concentration involving solvent extractions or filtration prior to IMS purification.

A concentrated and purified sample prior to IMS concentration would be expected to improve the sensitivity of the assay. This assertion is supported by results from Lopez-Sabater *et al.* (1997) who investigated the use of IMS to assist purification of HAV from oysters. Those authors reported detection of as few as 10 PFU HAV in 20 g shucked oyster using RT-PCR in a protocol in which virions were concentrated 500-fold by a combination of ultrafiltration and IMS concentration. In the method described in this chapter, virions were concentrated 20-fold by the IMS method. Thus, the incorporation of an analogous secondary concentration procedure that concentrated HAV in the sample a further 25-fold, would be expected to increase the procedure's sensitivity (i.e. ≤ 138 TCID₅₀/10g) to at least that reported by Lopez-Sabater *et al.* (1997).

Future improvements to the protocol may use oligonucleotide probes that only fluoresce when bound to a specific DNA sequence during PCR amplification. This results in a reaction of high specificity compared to one employing SYBR Green dye, which fluoresces when bound to any double-stranded DNA (including non-specific products).

Expansion of this technique may be possible for detection of viral contamination in foods or environmental samples, by using IMS with antibodies specific for a number of viruses. A multiplex PCR could then be performed to detect a number of different viruses in the one reaction, giving a rapid and specific analysis of the type of contamination present. The use of monoclonal antibodies primed to an epitope on viral capsids that is essential for attachment to host cells, i.e. in initiating infection is recommended. If the antibody cannot bind to this epitope due to protein denaturation on the capsid, it is likely the virion's infectivity will also be diminished.

Final Discussion

Enteric viruses, and particularly HAV and human norovirus, present a unique challenge to public health and food safety authorities. They can survive for long periods of time in the environment, and may still be infectious when other markers of human faecal waste are undetectable. Infection is often spread rapidly between humans due to the low infectious dose of the viruses, coupled with their stability in foods and on surfaces, especially at refrigerated and freezing temperatures.

Foods that undergo mild cooking or do not require cooking are those most often implicated in the transfer of enteric viral infection. Filter-feeding shellfish, and oysters in particular, have historically been linked to many outbreaks of enteric viral illness due to their ability to accumulate and concentrate viruses from harvest water contaminated with human faeces. This is despite constant improvements to wastewater treatment processes and updated water and shellfish monitoring programs.

HPP has been of great interest to shellfish processors, mainly because the process shucks oysters cleanly to produce a high quality product. When applied appropriately, it also extends the refrigerated shelf life of oysters by a number of weeks without adversely affecting the fresh organoleptic and nutritional properties of the oyster, opening marketing and export opportunities for a high end, value-added product.

Predictably, interest has been generated in examining the effect of HPP on enteric viruses, and the research performed to date has provided important inactivation data for a number of viruses, both in buffered media and in food matrices. HPP inactivation data generated for HAV to date has been generated from a range of pressure, time and temperature combinations that are difficult to compare, since each of these variables affects the microbial sensitivity to high pressure. For example, the effect of high pressure on microorganisms varies with different substrates and their salinity, and with different initial treatment temperatures. If meaningful comparisons are to be drawn between high pressure treatments, then it is vital that all of these variables remain constant. Gaps in the knowledge surrounding the interactions of these variables on the sensitivity of HAV to HPP were addressed in this project, with a dataset generated and modelled to ensure predictions could be made throughout the dataset by interpolation.

A suitable method for generating high titre HAV stock was important, because growth of HAV is time consuming, and with a large amount of virus required for replicate HPP trials, the method used to generate virus stock needed to be easy and rapid to perform. This outcome was achieved by using the freeze-thaw method. Reasonably high titres of approximately 10^6 TCID₅₀/ml were achieved, without the need for ultracentrifugation, filtration or solvent extraction. Highly purified stocks of viruses are not necessarily beneficial during challenge testing, as viruses tend to aggregate and become associated with particulate matter in the environment.

The BSC-1 cells used in this study did not plaque or show indicators of cytopathic effects (CPE) during HAV infection; instead infection was persistent, with cell differentiation continuing as HAV progeny were released from cells over time. Presumably, a persistently-infected cell line could therefore be generated, with infected cells passaged at regular intervals while the medium is collected to generate virus stock.

Further modification to the freeze-thaw protocol may be required to increase the cultivated titre, and this could be achieved by simply increasing the size of tissue culture flasks in which inoculation of the cell monolayer is performed, or increasing the length of incubation time between cells and virus. In the case of increasing the length of incubation, cell stability may be affected. If the time between cell passage is too long, modification of media formulation may be required to ensure cell stability. Other strategies such as ultrafiltration or PEG precipitation of the virus stock would assist in concentrating viruses, but would lengthen the protocol and may result in the monodispersion of viruses, which is not always desired.

The investigation and development of a suitable method for recovering viruses from oyster tissue highlighted the variability in recovered titres that has been reported by other researchers. Modifications made to the PEG precipitation method were relatively minor, to ensure the time taken to complete the overall method was not increased substantially. In general, the variability was observed in all methods applied, which meant the overall difference between the titres recovered from each method did not appear substantial. The crude extraction method was preferred for use because the recoveries obtained were consistently higher than 40%, and the method itself was fast and simple to perform.

No concentration step was used in the crude extraction procedure. Furthermore, <1 ml of the 40 ml extract recovered was used for quantitation, so there is scope to improve detection by concentrating the extract and/or by increasing the volume of extract used in quantitation. However, the use of PEG precipitation for concentration was not beneficial to higher recovery in this study, and furthermore the length of the procedure was increased beyond a length acceptable for use in a challenge study with a large number of samples to be processed simultaneously. Quantitation by plaque assay (if possible with the HAV strain) with plates of larger surface area and requiring a larger inoculum volume, may increase sensitivity.

Molecular biological techniques such as RT-PCR are popular for the detection of viral genetic material in food and environmental samples. These samples also contain substances inhibitory to PCR that must be removed prior to the assay to ensure sensitivity of detection. The lack of purification steps in the crude extraction technique mean that to ensure sensitive detection of HAV RNA, an additional purification procedure during viral RNA extraction must be performed, and must be effective at removing these inhibitors. The Qiagen method of RNA extraction appeared suitable for this purpose in the Chapter 5 study of whole oysters following accumulation of HAV, with HAV RNA detected in samples below the detection limit of the infectivity assay. In the Chapter 7 study, however, no HAV RNA was detected within inoculated oyster homogenates using the Qiagen method. The smears seen in the gel indicate the presence of degraded nucleic acid which could be masking any HAV-specific band that was amplified during the reaction.

The inability to detect HAV RNA in artificially-inoculated oyster homogenate indicates the need for more sample processing prior to analysis. Sample processing may include purification of virions during the recovery process, or additional purification of viral RNA after extraction from virions. Either way, the processing required to obtain a higher level of purity increases the length of the process. It also increases the likelihood of nucleic acid loss, the effects of which become increasingly important as the level of viral contamination in a sample becomes smaller. Finding a balance between a highly purified sample, and one with a high sensitivity may well be the key in molecular detection of enteric viruses from foods. Use of RT-PCR protocols that are more robust with a higher

specificity to the target of interest will be vital for achieving optimal detection in food and environmental samples. For example, the use of SYBR Green dye in qRT-PCR for this purpose is not recommended due to its lack of sensitivity. The dye emits bright fluorescence when bound to any double-stranded DNA. For this reason, sequence-specific fluorogenic oligoprobes such as TaqMan are popular, where a single-stranded oligonucleotide labelled with a fluorophore and a fluorescence quencher binds to the complementary sequence on the DNA template. Fluorescence generated by the fluorophore is absorbed by the quencher molecule in the probe's natural conformation due to the close proximity of the two, but during DNA synthesis the polymerase enzyme degrades the oligonucleotide attached to the template, releasing the fluorophore and quencher separately into the solution, meaning the fluorescence is no longer quenched. The fluorescence generated by these fluorophores is therefore proportional to the amount of sequence-specific DNA amplicon in the reaction, and as a result, a melt curve is not strictly necessary with each run.

A repeated criticism of qRT-PCR is its inability to distinguish between RNA from infectious virions with naked RNA or RNA protected within a non-infectious capsid. The use of IMS in recovery trials with oyster homogenate ensured only virions with intact target epitope on the viral capsid were isolated, with an increased likelihood that the RNA detected is from an infectious virion. Absolute confirmation of infectivity is not possible, however, because the antigenic epitope targeted by the antibody is not necessarily the same site on the capsid that is in contact with the host cell receptor to initiate infection. While the method does not

give a definitive indication of infectious virus, it at least ensures free RNA won't be detected, and that a portion of the viral capsid will be intact.

The development of a method to detect infectious virus by RT-PCR should be a current priority in virology laboratories with the relevant expertise, and may be possible if, for the purposes of using IMS, monoclonal antibodies primed to an epitope on the virus capsid essential for infection were used, e.g. the site of attachment between virion and host cell receptor. Alternatively, a method such as integrated cell culture (ICC) RT-PCR, described by a number of researchers in recent years to detect infectious virus may be used. The method involves infecting a cell culture with recovered virus, followed by detection of virus progeny in the cell culture medium (Murrin and Slade, 1997; Reynolds *et al.*, 2001; Greening *et al.*, 2002; Bhattacharya *et al.*, 2004). The benefit of such a method is the confirmation of infectious virus may be given in 2-4 days, rather than the 7 days taken in the infectivity assay used currently. An alternative method which alleviates the possibility of detecting non-infectious virions present in the cell culture media was described by Chaves *et al.* (1994) and Jiang *et al.* (2004), where negative-strand DNA is detected in infected tissue culture cells, proving that replication of the RNA virus has proceeded in the cell. A quantitative PCR-based method has not yet been described in the scientific literature, and would be particularly useful in determining virus sensitivity after processing.

The study described in Chapter 5 highlighted the difficulty that may be faced in contaminating live filter-feeding shellfish with virus in the laboratory. The uptake of virus was confirmed, but not to a titre high enough for challenge testing with HPP. The accumulation and concentration of enteric viruses to high titres within oysters has been observed by other researchers as described within this thesis, both in the environment and in the laboratory. In the Chapter 5 study, oysters were shown to have accumulated HAV from the surrounding seawater, but to a concentration that was too low for use in the HPP challenge study.

To increase virus accumulation by oysters, virions were first mixed with microalgae to promote binding, so that the increased particulate size would be more easily trapped by oysters than monodispersed virions. It became clear during the trial that the algae migrate to solid surfaces within the aquarium, including the oyster shell, tank walls and tubing. Furthermore, previous research has also indicated the propensity of free viruses to become attached to these same surfaces (Bedford *et al.*, 1978). The result of these observations appears to be the removal of viruses from the water, meaning oysters are no longer able to take them up, and probably explained the low amount of uptake within oysters observed in the current study. Despite these findings, regular observations made throughout the trial indicated the oysters' valves were open, and that faeces and pseudofaeces were being produced by all oysters examined.

It appears that laboratories equipped with flow-through aquarium systems capable of supplying a constant flow of sterilized seawater have a significant advantage over the recirculating aquarium, due to the ability to supply a constant supply of algae and virus as well as water, keeping the environmental conditions within the aquarium constant. Future investigation of virus accumulation should begin with confirming the location and the infectious state of the virions unaccounted for in the contaminated water and solid surfaces within the aquarium during the accumulation trial. Once the location of virions within the aquarium are known, improvements to the contamination method can be carried out, and may involve the addition of substances such as BSA into the water (Bedford *et al.*, 1978) to reduce the adsorption of virions to the walls of the aquarium. The association of viruses with microalgae may even enhance this adsorption, and addition of host cell-associated virus to the water may ensure a higher uptake by oysters is achieved.

The method of accumulation described in Chapter 5 was investigated as a way to contaminate oysters prior to HPP treatment. These treatments were designed to evaluate the predictions of the developed model for HAV inactivation due to HPP in buffered media. Due to the inability of achieving a high enough level of accumulation, oyster homogenate was artificially inoculated with a known titre of HAV prior to high pressure treatment. Viruses suspended within oyster homogenate were expected to be representative of those naturally accumulated by oysters in terms of their sensitivity to high pressure. Virus stock added to homogenate was visibly 'soaked up' into the homogenate, which was thought to represent the adsorption of viruses to the oyster particulates.

The effect of high pressure on viruses in buffered media, as reported in Chapter 6 and like those reported in past studies, indicated that curvature exists in the inactivation curve of HAV when suspended in buffered media and treated with HPP. Unlike previous studies, however, the curvature described by the Weibull model was not consistently a tailing shape. The erratic behavior of HAV inactivation meant that the aim of the study, to develop a secondary model describing HAV inactivation by HPP, was not possible with the Weibull function, and was instead achieved with the log-linear equation (equation 7) in Chapter 6. The developed log-linear model can now be expanded and modified with the introduction of additional data. The generation of additional HPP inactivation data within the range of pressures tested during these studies is recommended to help explain the erratic behaviour observed in the inactivation curves at different pressure and salinity combinations.

The model was found to be fail-safe in many of its predictions during evaluation in artificially-inoculated homogenate, especially during treatments at >375 MPa. The oyster homogenate was therefore contributing to greater inactivation in comparison to buffered media; an unexpected result, considering the protection from environmental stress and to disinfection procedures that is usually afforded to virions by adsorption to particulates. The exposure of viruses to normally intra-cellular enzymatic processes after homogenization and disruption of cells may have contributed to capsid damage, although measures such as refrigeration of samples were taken to minimize the extent of this effect.

The results of high pressure treatment presented within these studies may be replicated in the future, on HAV in naturally-contaminated whole oysters, to determine any additional effects that homogenization of the oysters may have had on the sensitivity of HAV to high pressure. In the meantime, both the log-linear secondary model described in equation 7 (Chapter 6), and the six individual Weibull primary models valid at the specified pressure and salinity combinations in Chapter 6, are suitable to use as a reference tool during oyster processing by high pressure.

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Appendix

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Grove, S. F., Lee, A., Lewis, T., Stewart,
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